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Evaluation of red blood cell *Pig-a* assay and PIGRET assay in rats using chlorambucil

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ABSTRACT

The *Pig-a* assay is a novel method to assess the *in vivo* mutagenicity of compounds, and it is expected to be useful for the detection of genotoxicity. In this study, to assess the performance of the *Pig-a* assay targeting red blood cells (RBCs; RBC *Pig-a* assay) and reticulocytes (RETs; PIGRET assay), chlorambucil, which is a genotoxicant, was orally administered to male rats once at 10, 20 and 40 mg/kg on Day 1, and the mutant frequencies (MFs) of RBCs and RETs were examined periodically. In the RBC *Pig-a* assay, significant increases in MFs were observed at 40 mg/kg on Day 15 and at 20 mg/kg or higher on Day 29. In the PIGRET assay, MFs increased significantly at all dose levels on Day 8 and only at 20 mg/kg on Day 15, but there was no increase in MFs in the treatment groups on Day 29. In conclusion, the RBC *Pig-a* assay and PIGRET assay in rats have sufficient sensitivity to detect the mutagenicity of chlorambucil, and the PIGRET assay could detect its mutagenicity earlier and at a lower dose than the RBC *Pig-a* assay.

1. Introduction

The phosphatidylinositol glycan complementation group A (*Pig-a*) assay is a new *in vivo* mutation assay to assess the mutagenicity of compounds based on the presence or absence of glycosylphosphatidylinositol (GPI)-anchored proteins (CD59, CD24, CD48, *etc.*), and it is expected to be useful for the detection of genotoxicity [1,2]. *Pig-a* codes for a catalytic subunit of an *N*-acetylglucosamine transferase necessary for the synthesis of GPI anchors [3,4]. The *Pig-a* gene is located on the X-chromosome, and a single mutation leads directly to a mutant phenotype [5]. Thus, *Pig-a* mutations cause a deficiency of GPI-anchored proteins on the cell membrane. The *Pig-a* assay targeting red blood cells (RBCs; RBC *Pig-a* assay) has been mainly investigated. The *Pig-a* assay requires only small amounts of blood and, thus, the mutant frequency (MF) in the same animal can be assessed at multiple time-points. It can be conducted in various species including rats, mice and humans because the *Pig-a* gene is highly conserved among them. Moreover, its flow cytometric scoring technique enables us to complete the measurement of MFs in cells in a single day, which is much faster than the conventional genotoxicity tests, such as the chromosomal aberration test and micronucleus test, which are standard battery tests described in the International Conference on Harmonization (ICH) S2 (R1) guideline (Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use) [6]. Kimoto et al. recently developed a *Pig-a* assay targeting reticulocytes (RETs), the PIGRET assay, in 2011 [7]. Other *Pig-a* assays targeting RETs were reported before then, and Bryce et al. and Phonethepswath et al. stained blood cells to distinguish RETs and mature RBCs with Thiazole Orange and SYTO 13, respectively [8,9]. Kimoto et al. used the anti-CD71 (a RET marker) antibody and magnetic beads and separated RETs from blood cells. This method has a comparable sensitivity to detect MFs in RETs as traditional methods and leads to an improvement in the throughput of the *Pig-a* assay targeting RETs.

A collaborative study by the Mammalian Mutagenicity Study (MMS) Group, which is a subgroup of the Japanese Environmental Mutagen Society (JEMS), was undertaken to evaluate the usefulness of the RBC *Pig-a* assay and PIGRET assay for the detection of genotoxicity and to compare the RBC *Pig-a* assay and PIGRET assay in terms of sensitivity and the time period required to detect significant increases in MFs using 24 chemicals, which include genotoxicants and non-genotoxicants. In the present study, we confirmed that both the RBC *Pig-a* assay and PIGRET assay could detect the genotoxicity of chlorambucil in a single-dose study as a part of a collaborative study.

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2. Materials and methods

2.1. Chemicals

Chlorambucil (CAS No. 305-03-3, Lot No. BCBM2499V) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) and was dissolved in 10% ethanol (KOKUSAN CHEMICAL Co., Ltd., Tokyo, Japan).

2.2. Animals, treatment and blood sampling

Crl:CD(SD) male rats (Charles River Japan Inc., Yokohama, Japan) were housed (2 or 3 animals per cage) under controlled temperature, lighting and relative humidity conditions (19–25 °C, 12-h light/dark cycle and 40–60%, respectively). Male animals only were used in this study as discussed in the summary paper of this collaborative study [12]. The animals were given a standard diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. They were assigned to groups (6 animals per group) using a stratified random grouping method based on their individual body weights.

Chlorambucil was orally administered (10 mL/kg) to 8-week-old rats once by gavage at 10, 20 and 40 mg/kg on Day 1. The three doses were determined on the basis of LD_{50} (76 mg/kg) in rats and high dose (15 mg/kg/day) in the 3-day repeated-dose study [10,11]. During the period from Days 1 to 29, clinical signs were observed for all animals. The body weights were measured on Days 1, 2, 8, 15, 22 and 29. Blood was collected via the jugular vein under light isoflurane anesthesia using syringes treated with EDTA solution before administration (Day -4) and on Days 8, 15 and 29. This study was reviewed by the Animal Care and Use Committee and approved by the head of the test facility (Approval No.: AC2014-28 and AC2014-29), and performed in accordance with the Guideline for the Animal Experiments, Research & Development Division, Toray Industries, Inc.

2.3. RBC Pig-a assay and PIGRET assay

The RBC *Pig-a* assay and PIGRET assay were performed according to the protocol described in the summary report of this collaborative study [12].

Briefly, RBCs were incubated with FITC anti-rat CD59 (a GPI-anchored protein) antibody (BD Biosciences, San Jose, CA, USA) and APC anti-rat HIS49 (an erythroid marker) antibody (BD Biosciences) at room temperature for 1 h. The cells were centrifuged at 1680 × *g* and resuspended with phosphate-buffered saline (PBS).

RETs were labeled with PE anti-rat CD71 (transferrin receptor; a RET marker) antibody (BD Biosciences) at 4 °C for 15 min. After washing with 1 × IMag Buffer (BD Biosciences), the cells were incubated with PE particles Plus-DM (BD Biosciences) at 4 °C for 15 min. CD71-positive cells were collected by a cell separation system with a BD IMagnet (BD Biosciences). The CD71-positive cells were labeled with FITC anti-rat CD59 antibody and APC anti-rat HIS49 antibody as described above. The cells were centrifuged at 1680 × *g* and resuspended with PBS.

The MFs in RBCs and RETs and the percentages of RETs were scored using a flow cytometer running CellQuest Pro software (FACSCalibur; BD Biosciences). The MFs in RBCs and RETs are expressed as the number of CD59-negative cells per 1 million HIS49-positive RBCs and the number of CD59-negative cells per 1 million CD71 and HIS49 double-positive RETs, respectively. The percentages of RETs are expressed as the number of CD71-positive cells per 20 thousand HIS49-positive RBCs.

2.4. Statistical analyses

Statistical analyses of the MF data were performed at Teijin Pharma Limited using EXSUS Ver. 7.7.1 (CAC EXICARE Corporation, Tokyo, Japan) as follows. An offset of 0.1 was added to each MF value, because MFs of zero were occasionally observed, and then log (10) transformed. Transformed MF values were analyzed by Bartlett's test for homogeneity of variance among the groups. If the group variance was determined to be homogeneous, Dunnett's multiple comparison test was employed. If Bartlett's test indicated heterogeneous variance, the nonparametric Dunnett's multiple comparison test (Steel test) was used. Significance was evaluated at the 5% level using a one-tailed test for increases relative to the vehicle control.

The body weight and percentage of RETs were analyzed statistically using SAS Ver. 9.3.3 software (SAS institute Japan, Tokyo, Japan). The significance of differences compared to the control group was tested with Dunnett's multiple comparison.

3. Results

3.1. Clinical signs and body weight

No animal died in any group, and no changes in clinical signs attributed to chlorambucil were observed. The body weight decreased significantly and dose-dependently at 20 mg/kg or higher on Day 2, but no significant difference was observed between any chlorambucil-treatment group and the vehicle control group on or after Day 8 (Fig. 1).

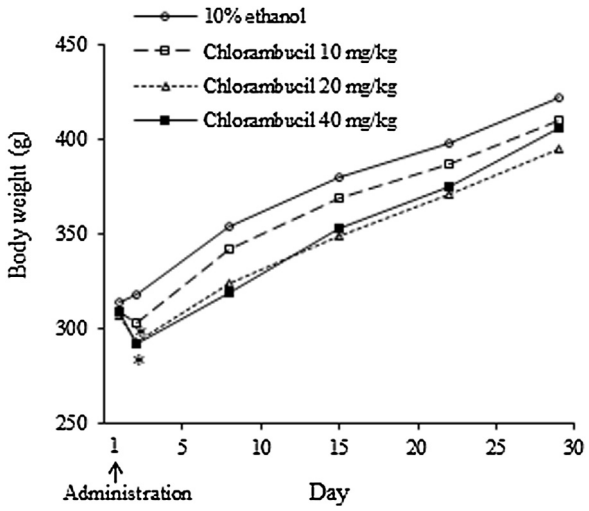

Fig. 1. Body weight after the administration of chlorambucil at 10, 20 and 40 mg/kg to 8-week-old rats. The data are presented as the mean of 6 animals in each group. Statistical analysis was performed using Dunnett's multiple comparison (*$P < 0.05$).

3.2. RBC Pig-a assay

The results of MFs in RBCs are shown in Table 1 and Fig. 2A. Significant increases in MFs were observed at 40 mg/kg on Day 15, and at 20 mg/kg or higher on Day 29, and MFs at 20 mg/kg or higher continued to increase until Day 29.

3.3. PIGRET assay

The results of MFs in RETs are shown in Table 2 and Fig. 2B. The MFs increased significantly at 10 mg/kg or higher dose levels on Day 8. On Day 15, MFs showed an increasing tendency at 10 mg/kg, and a significant increase at 20 mg/kg; however, MFs began to decrease at 40 mg/kg in spite of the largest increase on Day 8. No significant difference in MFs was observed in any chlorambucil-treatment group compared with the vehicle control group on Day 29. In one of six animals in the 40 mg/kg group, the PIGRET assay could not be conducted on Day 8 because the number of RETs was too small to evaluate it.

3.4. Percentage of RETs

The percentages of RETs are shown in Table 3 and Fig. 2C. The percentages of RETs in all chlorambucil-treatment groups were not significantly different from those in the vehicle control group. However, a decreasing tendency of RETs was observed at 40 mg/kg on Day 8, and they decreased to only 1.1% in one of the six animals of the group (vehicle control group: 6.9 ± 0.9%). On the other hand, the slightly increasing tendencies of RETs were detected at 10 and 20 mg/kg on Day 8. In all groups, the percentages of RETs were lower on Days 15 and 29 than those at pre-dosing.

4. Discussion

Chlorambucil is a rodent genotoxic carcinogen targeting lung, hematopoietic system, mammary gland and nervous system [13–16]. Chlorambucil is a nitrogen mustard that acts as a chloroethyl alkylating agent and is used widely in the treatment of human cancers [17].

It was reported that repeated dosing (3 and 28 days) of chlorambucil induced MFs in RBCs and RETs at 2 weeks after the beginning of dosing, and MFs in RETs increased much earlier than RBCs [10,11]. However, the measurement of MFs at 1 week after