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Evaluation of the RBC *Pig-a* and PIGRET assays using single doses of hydroxyurea and melphalan in rats

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ABSTRACT

To evaluate the suitability of the rat *Pig-a* assay on reticulocytes (PIGRET assay) as a short-term test, red blood cell (RBC) *Pig-a* and PIGRET assays after single doses with hydroxyurea (HU) and melphalan (L-PAM) were conducted and the results of both assays were compared. HU was administered once orally to male SD rats at 250, 500 and 1000 mg/kg, and both assays were conducted using peripheral blood withdrawn from the jugular vein at 1, 2 and 4 weeks after dosing. L-PAM was administered at 1.25, 2.5 and 5 mg/kg in the same manner. L-PAM produced significant dose-dependent increases in mutant frequencies in the PIGRET assay after single oral doses, but did not produce dose-dependent increases in mutant frequencies in the RBC *Pig-a* assay. These results suggest that the PIGRET assay is more sensitive for the evaluation of the mutagenic potential of L-PAM than the RBC *Pig-a* assay. In contrast, HU, a clastogenic but not DNA-reactive compound, gave negative results in both assays. The results with these 2 chemicals indicate that the single-dose PIGRET assay in rats has the potential to properly detect DNA-reactive compounds that directly cause DNA damage in a short-term assay.

1. Introduction

The X-linked *Pig-a* (phosphatidylinositol glycan, Class A) gene codes for the catalytic subunit of an *N*-acetylglucosamine transferase, which is involved in glycosylphosphatidylinositol (GPI) anchor synthesis [1]. The *Pig-a*-defective mutations abolish GPI synthesis, resulting in a deficiency of GPI-anchored protein markers (e.g., CD59) expressed on the cell surface. An *in vivo Pig-a* gene mutation assay with flow cytometric analysis for detecting GPI-negative cells in rat peripheral blood (RBC *Pig-a* assay) was established [2,3]. The *Pig-a* assay requires only a small amount of blood sample and is able to evaluate the mutant frequencies (MFs) over time in a less invasive manner than the transgenic rodent mutation assays. An expert workgroup formed by the International Workshop on Genotoxicity Testing (IWGT) indicated a growing interest in developing a practical method for measuring *in vivo* gene mutation as an alternative to the existing transgenic rodent mutation assays and considered integrating it into a 28-day repeated dose general toxicity test [4]. The *Pig-a* assay is now anticipated to be issued in a new Organization for Economic Co-operation and Development (OECD) test guideline.

Kimoto et al. have developed the PIGRET assay, in which reticulocytes (RETs) were selectivity enriched from peripheral blood by magnetic separation of cells positive for CD71, a transferrin receptor expressed on the surface of RETs but not on the surface of mature red blood cells (RBCs) [5]. The apparent increases in RET *Pig-a* MFs were detected 1 week after treatment with a single dose of *N*-ethyl-*N*-nitrosourea (ENU). At this sampling time, the frequency of CD59-negative RBCs was still quite low. The authors speculate that mutated cells transit from the bone marrow and slowly accumulate in the peripheral blood mainly as RETs, resulting first in an increase in the RET *Pig-a* MF and later in the RBC *Pig-a* MFs.

In Japan, an inter-laboratory trial of the rat RBC *Pig-a* and PIGRET assays was performed [6]. It was notable that the PIGRET assay consistently detected increases in *Pig-a* MFs by 4-nitroquinoline-1-oxide and 7,12-dimethylbenz[a]anthracene 1 week after a single dose, whereas the RBC *Pig-a* MFs were weakly increased at this sampling time. Therefore, the PIGRET assay may detect the *in vivo* mutagenicity of test compounds at an earlier time point after dosing than the RBC *Pig-a* assay.

To verify the usefulness of the PIGRET assay as an *in vivo* short-term genotoxicity test, a collaborative study of the RBC *Pig-a* and PIGRET assays after single doses by the Mammalian Mutagenicity Study (MMS) Group of The Japanese Environmental Mutagen Society (JEMS) was started in 2013. The other purpose of this collaborative study was to submit information on the usefulness of

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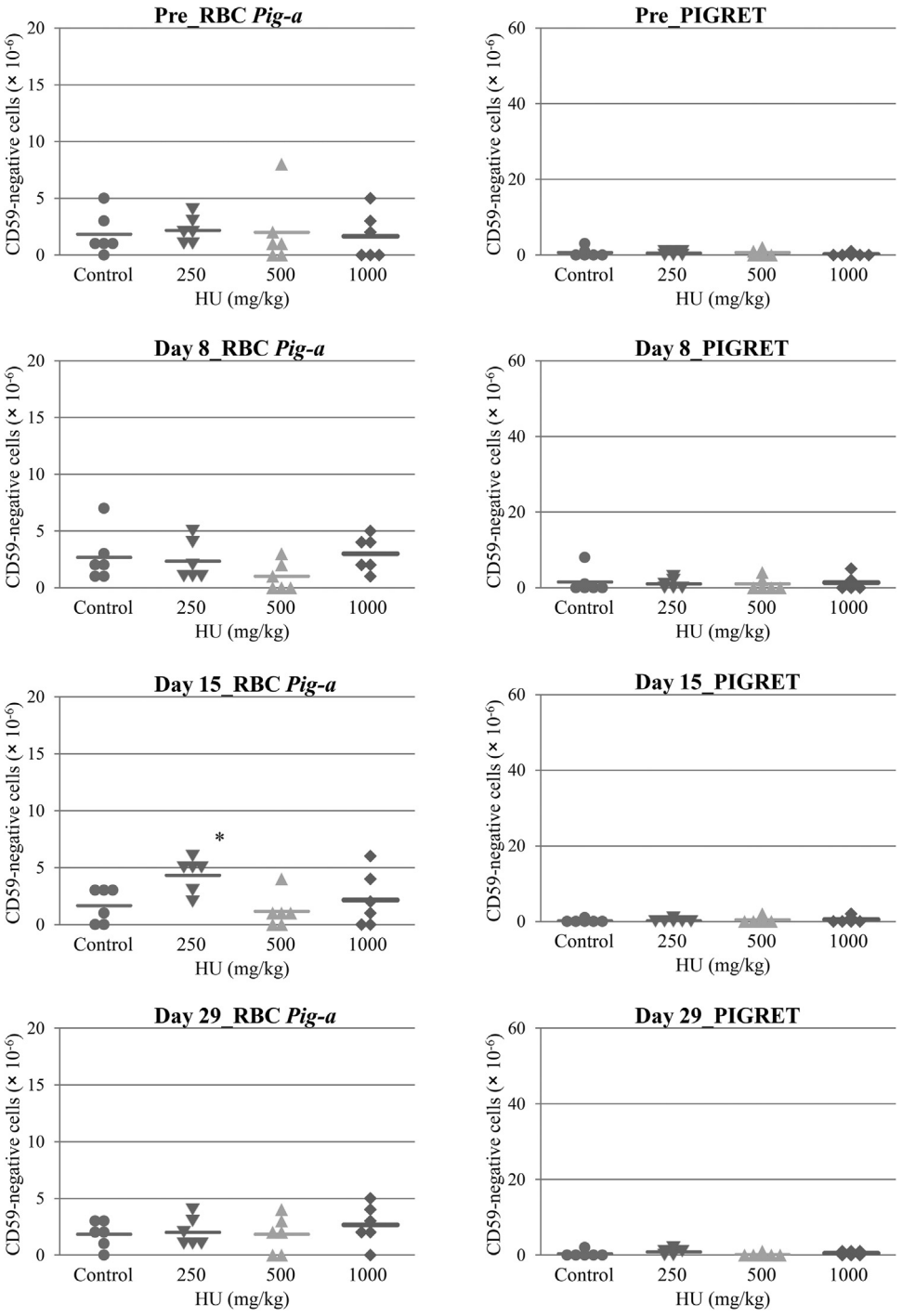

Fig. 1. *Pig-a* mutant frequencies in rats treated with a single dose of HU (n = 6). RBC *Pig-a* and PIGRET assays before (Pre) and 7, 14, and 28 days after the administration (Day 8, 15 and 29, respectively) were conducted. All individual data are shown as plots, and a bar indicates the mean of each group. Statistically significant differences from the respective vehicle controls are indicated at the $p < 0.05$ levels (*).

the PIGRET assay to the OECD for establishment of the OECD test guideline.

As a part of the collaborative study organized by the 16 participant laboratories for a single administration using the RBC *Pig-a* and PIGRET assays, the participant laboratories each assessed 1 or 2 chemicals from 24 chemicals whose genotoxic activity was known, which had previously been tested in a repeated-dose RBC *Pig-a* assay, and the results were reviewed by the expert workgroup formed by IWGT [4]. To evaluate the suitability of the PIGRET assay as a short-term test, we conducted the RBC *Pig-a* and PIGRET assays after single oral doses with hydroxyurea (HU) and melphalan (L-phenylalanine mustard, L-PAM) and compared the results of both assays. L-PAM is a known mutagen, while HU is a clastogen but not a DNA-reactive compound [7,8].

2. Materials and methods

This study consisted of dose-finding and main tests, and the main tests were carried out once each for evaluation of HU or L-PAM.