Original Article

Effective use of the *Pig-a* gene mutation assay for mutagenicity screening: measuring CD59-deficient red blood cells in rats treated with genotoxic chemicals

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(Received June 10, 2012; Accepted July 11, 2012)

ABSTRACT — The *Pig-a* gene mutation assay using peripheral blood erythrocytes is being investigated as a screening tool for assessing mutagenicity *in vivo*. In this study, we evaluated two distinct approaches for performing the *Pig-a* assay in rats. We used antibodies to CD45 or the erythroid marker HIS49 to identify red blood cells (RBCs), and then monitored the kinetics of *Pig-a* mutant frequency, as measured by the frequency of CD59-deficient RBCs, in rats treated with the genotoxic chemicals, *N*-ethyl-*N*-nitrosourea, cyclophosphamide, 4-nitroquinoline-1-oxide, and ethylmethanesulfonate. In some instances, micronucleus frequency also was measured in the same animals. Time- and dose-related increases in *Pig-a* mutant frequency were found in all the chemical-treated groups, except for the groups treated with cyclophosphamide, which was a potent inducer of micronuclei. The two different approaches we employed were comparable for measuring induced mutant frequencies, but our historical data showed that the mean background frequencies for the CD45/CD59 method and the HIS49/CD59 method were 12.7×10^{-6} and 5.5×10^{-6} , respectively. The relatively low, stable background mutant frequency associated with the HIS49/CD59 method indicates that it may have greater power for discriminating weak induced responses. These results suggest that the HIS49/CD59 method is a promising tool for measuring *Pig-a* mutant RBCs. In addition, differences in their manifestation kinetics and in their relative sensitivity for detecting different test compounds suggest that the combination of the *Pig-a* assay and the micronucleus assay may be effective in identifying *in vivo* genotoxicity.

Key words: *In vivo* gene mutation assay, *In vivo* micronucleus assay,
Phosphatidylinositol glycan complementation class A gene, Flow cytometry,
Peripheral red blood cells

INTRODUCTION

Chemicals that induce genetic damage to DNA (e.g., gene mutation and chromosome aberration) may cause cancer and/or heritable defects (Duesberg *et al.*, 2005). Both *in vitro* and *in vivo* genotoxicity tests have been established to assess the genotoxicological risk of chemicals, including candidate pharmaceuticals (Kirkland *et al.*, 2005; Rothfuss *et al.*, 2011). *In vivo* tests fall into three general categories, those that measure clastogenicity and possibly aneugenicity, those that measure gene mutation, and those that measure DNA damage. The micronucleus test, which can evaluate the ability of chemicals to induce structural and numerical chromosome aberration,

is the primary *in vivo* genotoxicity test used in regulatory science (Tweats *et al.*, 2007). In particular, remarkable developments have been made in flow-cytometric scoring of micronucleated reticulocytes (MN-RET) in peripheral blood (PB) as a screening assay (Dertinger *et al.*, 1996, 2011a). The Comet assay, which can evaluate the ability of chemicals to induce primary DNA damage, has a validated protocol (Hartmann *et al.*, 2003), and is undergoing Organization for Economic Co-operation and Development (OECD) Test Guideline development. It is cited as a second *in vivo* test by the International Conference on Harmonization (ICH) S2(R1) guideline for further assessing the *in vivo* genotoxicity of human drugs (ICH S2(R1), Current step 4 version dated

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9 November 2011). The gene mutation assays conducted with transgenic animals and the Hypoxanthine phosphoribosyltransferase gene (*Hprt*) lymphocyte assay have been used worldwide to assess the risk of *in vivo* gene mutation (Casciano *et al.*, 1999; Thybaud *et al.*, 2003). However, these tests are considered impractical for routine use and no *in vivo* gene mutation assays are included in the ICH S2(R1) battery.

In recent years, a novel *in vivo* gene mutation assay has been developed that uses the phosphatidylinositol glycan, class A gene (*Pig-a* in rodents, *PIG-A* in humans) as an endogenous reporter (Dertinger and Heflich, 2011; Dobrovolsky *et al.*, 2010a; Peruzzi *et al.*, 2010). The principle of the assay is that the pathway for glycosylphosphatidylinositol (GPI) anchor synthesis requires the *Pig-a* gene product; thus the absence of GPI-anchored proteins on the surface of mammalian cells (usually erythrocytes) can be used as a reporter of *Pig-a* mutation. The *Pig-a* assay has several advantages that recommend it as a prediction tool for *in vivo* somatic mutation. One is that results can be generated in only hours using flow cytometry, with no cell culture necessary. Another is that the *Pig-a* assay can be used for measuring mutation in various mammals, including rodents and humans, because the gene is highly conserved (Kawagoe *et al.*, 1994; Phonethepswath *et al.*, 2008; Takeda *et al.*, 1993). While the assays that measure mutation in rodent erythrocytes have benefited from the most developmental effort, technical trials of the red blood cell (RBC) *PIG-A* assay in humans and in non-human primates have demonstrated their feasibility for measuring mutation in these species (Dobrovolsky *et al.*, 2009, 2011).

Previous studies have progressively clarified the characteristics of the *Pig-a* assay. During our efforts to develop a flow cytometric *Pig-a* assay for measuring gene mutation in *N*-ethyl-*N*-nitrosourea (ENU) treated rodents, we demonstrated increased frequencies of GPI-anchored-protein-deficient peripheral RBCs, bone marrow erythroids, and splenocytes (Kimoto *et al.*, 2011a, 2011b; Miura *et al.*, 2008a, 2008b). The use of non-nucleated RBCs to measure *Pig-a* gene mutation has been supported by sequence analysis of the *Pig-a* gene in GPI-anchored-protein-deficient bone marrow erythroids, which are the presumed precursors of PB RBCs (Kimoto *et al.*, 2011b). Related to this, we have proposed a model for *Pig-a* mutant manifestation to account for the accumulation and persistence of GPI-deficient *Pig-a* mutant RBCs observed in ENU treated rats (Miura *et al.*, 2009). The *Pig-a* assay can be performed with microliter volumes of PB so that the integration of the assay into repeated-dose toxicology studies or other genetic toxicology studies is feasible, which is con-

sistent with spirit of the 'three Rs' (Dertinger *et al.*, 2010; Phonethepswath *et al.*, 2010). In addition, the methodology for measuring *Pig-a* mutant cells developed by Dertinger and his colleagues was found to be reproducible and transferable in an international *Pig-a* collaborative study (Dertinger *et al.*, 2011c). These dedicated efforts raise hope that the *Pig-a* assay may be an effective tool for evaluating *in vivo* mutagenicity.

There are various approaches for measuring RBC *Pig-a* mutant frequency in rats. Miura *et al.* (2008a) developed an assay that labels PB with fluorescent antibodies specific for the leukocyte common antigen, CD45, to exclude leukocytes from the analysis. Another approach is to first leukodeplete PB samples by gradient centrifugation and then stain for reticulocytes (RETs) with Thiazole Orange or SYTO 13, which binds nucleic acid (Bryce *et al.*, 2008; Phonethepswath *et al.*, 2008). This approach was used in the international collaborative study and made it possible to distinguish RETs from mature RBCs and to evaluate *Pig-a* mutant frequencies in both populations at the same time (Dertinger *et al.*, 2011c). While both scoring approaches described above excluded nucleated cells from the analysis based on leukocyte-associated fluorescence (i.e., anti-CD45 or high nucleic acid dye-associated signal), the approach developed by Dobrovolsky *et al.* (2010b) used the anti-rat erythroid marker, HIS49, to positively identify RBCs in PB. Enriching the erythroid population with HIS49 antibody facilitates performing the *Pig-a* assay as it evaluates *Pig-a* mutant frequency specifically in RBCs without the need to leukodeplete the blood. However, most of the available *Pig-a* mutant data has been produced using leukodepletion, and little is known about the relative merits of performing the RBC *Pig-a* assay using anti-CD45 or HIS49 antibodies.

In this study, we evaluated the performance of two distinct *Pig-a* assays, hereafter referred as the CD45/CD59 and HIS49/CD59 assays, as screening tools for *in vivo* mutagenicity. We used both the CD45/CD59 and/or HIS49/CD59 protocols to monitor the kinetics of *Pig-a* mutant frequency, as measured by the frequency of CD59-deficient RBCs in rats treated with standard genotoxic chemicals (ENU, cyclophosphamide (CP), 4-nitroquinoline-1-oxide (4-NQO), and ethylmethanesulfonate (EMS)). We also compared the kinetics of *Pig-a* mutant manifestation to that of micronucleus induction in rats treated with ENU or CP. Finally, we evaluated the comparability and relative sensitivity of the *Pig-a* mutant response produced in CD45-negative cells and HIS49-positive RBCs.

MATERIALS AND METHODS

Reagents

ENU, EMS, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-NQO and methylcellulose (50 cps) were purchased from Wako-Pure Chemical Industry Co., Ltd. (Tokyo, Japan). Cyclophosphamide hydrate (Endoxan®; CP) was purchased from Shionogi & Co., Ltd. (Osaka, Japan). Calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.2) was purchased from Invitrogen, (Carlsbad, CA, USA), while saline (Otsuka Normal Saline) was purchased from Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan).

Anti-rat CD59 antibody (clone TH9, FITC-conjugated), anti-rat CD45 antibody (clone OX-1, PECy5-conjugated), anti-rat erythroid marker (clone HIS49, biotin-conjugated), and streptavidin-APC were purchased from BD Biosciences (Tokyo, Japan). *In vivo* Rat MicroFlow® Kits, for flow cytometric micronucleus scoring, were purchased from Litron Laboratories (Rochester, NY, USA).

Animals, administration of chemicals and blood collection

All experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (Teijin Pharma Ltd., Tokyo, Japan), and each experimental protocol was approved by the Committee for Animal Experiments of the Teijin Institute for Biomedical Research. Male CD(SD) rats were obtained from Charles River Laboratories (Yokohama, Japan); the rats received feed and water *ad libitum*. Rats were administered test agents by gavage after a 7 to 12-day acclimation period.

A summary of the experiments conducted for this study is shown in Table 1. ENU was dissolved in warm (37°C) PBS (pH adjusted to 6.0–6.1) at a concentration of 10 mg/ml (ENU concentration adjusted to account for the water and acetic acid stabilizer in the commercial preparation), sterilized with a 0.2 µm filter, and diluted to 1.0 and 4.0 mg/ml. Rats were treated with four weekly doses of 0, 10, or 40 mg/kg ENU, or with single doses of 0 or 40 mg/kg. CP was dissolved in PBS (pH 7.2) at a concentration of 10 mg/ml and diluted to 0.5 and 2.0 mg/ml. Rats were treated with 10 daily doses of 0 or 5 mg/kg CP except for the weekend, or single dose of 20 mg/kg CP. 4-NQO was dissolved in 0.5% (w/v) methylcellulose in distilled water and diluted to 5.0, 10, and 20 mg/ml. Rats were treated with single doses of 0, 50, 100, or 200 mg/kg 4-NQO. EMS was dissolved in PBS (pH 7.2) and saline, in Experiment 1 and Experiment 2, respectively. EMS used in

Experiment 1 was diluted to 9.0 and 36 mg/ml. Rats were treated with four weekly doses of 0 or 90 mg/kg or with a single dose of 360 mg/kg EMS, respectively. In Experiment 2, EMS was diluted to 1.2, 2.0, 18, and 36 mg/ml. Rats were treated with 28 daily doses of 0, 12, or 20 mg/kg EMS, or with single doses of 180 or 360 mg/kg EMS, respectively. All preparations were administered to rats in a volume of 10 ml/kg.

Blood was collected from the tail vein of treated animals as described in Table 1. All PB was promptly mixed with an EDTA solution (9 parts blood to 1 part 12 mg/ml EDTA).

Pig-a assay

Two distinct staining strategies (CD45/CD59 method and HIS49/CD59 method) were conducted for this study. For the CD45/CD59 method, 1 µl of blood/EDTA mixture was suspended in 0.2 ml of PBS, and the cells were labeled with 1 µg of PECy5-conjugated anti-rat CD45 antibody and 1 µg of FITC-conjugated anti-rat CD59 antibody. After incubation for 1 hr in the dark at room temperature, the cells were washed with PBS, centrifuged 5 min at $1,680 \times g$, and resuspended in 1 ml of PBS. Flow cytometry (FCM) analysis of both CD45 and CD59-negative cells was conducted on a EPICS-XL flow cytometer equipped with a 488 nm blue laser (Beckman Coulter, Tokyo, Japan). Typical cytograms are shown in Fig. 1A. A single cell population was gated by light scatter, and then CD45-positive white blood cells were excluded from this population by gating on CD45-negative cells. Approximately 300,000 to 500,000 CD45-negative cells (presumed RBCs) were analyzed further for expression of CD59 on the cell surface. For the HIS49/CD59 method, 3 µl of blood/EDTA mixture were suspended in 200 µl of PBS, and the cells were labeled with 0.25 µg of biotinylated HIS49 antibody and 1 µg of FITC-conjugated anti-rat CD59. After incubation for 1 hr in the dark at room temperature, the samples were washed with PBS, centrifuged 5 min at $1,680 \times g$, and resuspended in 200 µl of PBS; 0.25 µg of APC-conjugated streptavidin were added, and the samples were incubated for 15 min in the dark at room temperature. The cell samples then were centrifuged 5 min at $1,680 \times g$ and resuspended in 1 ml of PBS. FCM analysis of HIS49-positive and CD59-negative RBCs was conducted on a FACS Aria™ I flow cytometer equipped with 405 nm violet, 488 nm blue, and 635 nm red lasers (BD Biosciences, Tokyo, Japan). Typical cytograms are shown in Fig. 1B. A single cell population was gated by light scatter, and then HIS49-positive RBCs were gated. Approximately 300,000 to 500,000 HIS49-positive RBCs were analyzed further for expres-

Table 1. Summary of experiments measuring *Pig-a* mutant and/or micronucleus frequencies in rats treated with genotoxic chemicals

Test agent ^{a)}	Dos ^{b)} (mg/kg)	Frequency of Dosing	Assays Conducted ^{c)}	Time of Blood Sampling
ENU	0	4 (Weekly)	<i>Pig-a</i> assay (CD45/CD59 method ^{d)}) Micronucleus test (MicroFlow Plus ®)	Pre and 1, 2, 3, 4, 6, and 8 weeks after the first dosing Pre and 2, 6, 9, 13, 16, 20, 23, and 27 days after the first dosing
	10			
	40			
CP	0	10 (Daily, except for weekend) 1 (Single)	<i>Pig-a</i> assay (CD45/CD59 method) Micronucleus test (MicroFlow Plus ®)	Pre and 1, 2, 3, 4, 6, 8, and 10 weeks after the first dosing Pre and 2, 15, and 22 days after the first dosing
	5			
	20			
4-NQO	0	1 (Single)	<i>Pig-a</i> assay (CD45/CD59 method) <i>Pig-a</i> assay (HIS49/CD59 method ^{e)})	Pre and 1, 2, 3, 4, 6, 8, and 10 weeks after the first dosing Pre and 1, 2, 3, 4, 6, 8, and 10 weeks after the first dosing
	50			
	100			
	200			
	40			
ENU	40	1 (Single, included as a positive control)		
EMS (Experiment 1)	0	4 (Weekly)	<i>Pig-a</i> assay (HIS49/CD59 method)	Pre and 2, 4, 6, and 8 weeks after the first dosing
	90			
	360			
EMS (Experiment 2)	0	28 (Daily)	<i>Pig-a</i> assay (HIS49/CD59 method)	Pre and 2, 4, 6, and 8 weeks after the first dosing
	12			
	20			
	180			
	360			

^{a)} ENU: *N*-ethyl-*N*-nitrosourea, CP: Cyclophosphamide, 4-NQO: 4-nitroquinoline-1-oxide, EMS: Ethylmethanesulfonate

^{b)} Dosing Route : Gavage

^{c)} Both the *Pig-a* assay and Micronucleus test were conducted using rat peripheral blood.

^{d)} CD45/CD59 method: Red blood cells were defined as CD45-negative cells in peripheral blood.

^{e)} HIS49/CD59 method: Red blood cells were defined as HIS49-positive cells in peripheral blood.

Fig. 1. Flow cytometry analysis for detection of CD59-negative RBCs. (A) For analysis with the CD45/CD59 protocol, cells first were gated by light scatter to exclude the platelets and then for the expression of CD45 (pan-leukocyte marker). CD45-negative cells (the presumed RBC population) were gated and analyzed for CD59 expression. (B) For analysis with the HIS49/CD59 protocol, cells first were gated by light scatter and then for the expression of rat erythroid marker, HIS49. HIS49-positive cells were gated and analyzed for CD59 expression.

sion of CD59 on the cell surface.

No fluorescence compensation was employed for either method. All gates for detecting CD59-negative mutant RBCs were set using unstained and single-antibody-stained samples before evaluating the multi-antibody-

labeled experimental samples. As described by Kimoto *et al.* (2011a), the frequency of CD59-negative RBCs in antibody-stained blood samples was estimated from the number of CD59-negative RBCs falling into the mutant gate and the total number of RBCs interrogated.

Micronucleus assay

Micronucleus assays using rat PB samples were conducted with *In vivo* Rat MicroFlow® Kits. All samples were prepared according to the instruction manual in the kit. Approximately 20,000 CD71-positive RETs were interrogated for the frequency of micronuclei-positive RETs (MN-RET%) and CD71-positive RETs (%) using a FACSaria™ I flow cytometer (BD Biosciences).

Statistical analysis

Dunnett's multiple comparison t-test or Student's t-test was used for the statistical analysis. For the ENU, 4-NQO and EMS (Experiment 2) study, Dunnett's multiple comparison t-test was used for comparisons between the vehicle control group and each of the treatment groups at each time point. For the CP and EMS (Experiment 1) study, Student's t-test was used for comparisons between the control group and each treatment group at each time point because there were two treatment groups with different numbers of doses. Levels of significance were expressed as $p < 0.05$, $p < 0.01$, or $p < 0.001$.

RESULTS

ENU: Monitoring *Pig-a* mutant kinetics and micronucleus induction

In the ENU experiment, rats were administered 0, 10, or 40 mg/kg ENU in four weekly doses, and *Pig-a* mutant frequency was measured as the frequency of CD45-/CD59- RBCs before dosing (Pre), and at 1, 2, 3, 4, 6, and 8 weeks after the first dosing. In addition, MN-RET% and the percentage of CD71-positive RETs among total erythrocytes were scored at Pre, 2, 6, 9, 13, 16, 20, 23, and 27 days after the first dosing. Transient decreases in CD71-positive RETs (%) were found 2 days after each dose of 10 and 40 mg/kg ENU, compared to that of vehicle control (Fig. 2A). Dose related increases in *Pig-a* mutant frequency were found in the ENU treatment groups, and these mutant frequencies increased steadily over the 8 week observation period (Fig. 2B). Micronucleus induction in the same ENU treated rats was increased at 2 days after the each treatment, but then diminished to near control levels within 1 week after the treatments. Thus, the kinetics of the response and the persistence of the response were different for *Pig-a* mutant frequency and micronucleus induction (Fig. 2B).

CP: Monitoring *Pig-a* mutant kinetics and micronucleus induction

In the CP experiment, rats were administered either

Fig. 2. Percent of CD71-positive RETs among total RBCs (A) and the kinetics of micronucleated RET frequency (MN-RET%) and the frequency of CD59-negative RBCs (B) in rats treated with *N*-ethyl-*N*-nitrosourea (ENU). All data are the mean $\pm$ S.D. of data from five animals in each group. Dark arrows indicate the time of treatment. Doses of ENU were as follows: 0 mg/kg $\times$ 4 weekly doses (open circles), 10 mg/kg $\times$ 4 weekly doses (solid triangles), and 40 mg/kg $\times$ 4 weekly doses (open triangles). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$, 0.01, or 0.001 levels (*, **, or ***, respectively).

10 daily doses of 0 or 5 mg/kg CP except for the weekend, or a single dose of 20 mg/kg CP. *Pig-a* mutant frequency was measured as the frequency of CD45-/CD59- RBCs at Pre, 1, 2, 3, 4, 6, 8, and 10 weeks after the first dosing. In addition, MN-RET% and the percentage of CD71-positive RETs among total erythrocytes were scored at Pre and 2, 15, and 22 days after the first dosing. A transient decrease in CD71-positive RETs (%) was found 2 days after the single dose of 20 mg/kg CP, compared to that of the vehicle control (Fig. 3A). Also, a persistent decrease in CD71-

positive RETs (%) was found as a result of the repeated doses of 5 mg/kg CP. These decreases recovered to vehicle control levels at 1 or 2 weeks after the final dosing. Except at 4 weeks after the first treatment with 5 mg/kg CP, no significant increases in *Pig-a* mutant frequency were found at any of the sampling points (Fig. 3B). Micronucleus induction in the same CP-treated rats was increased at 2 days after the first treatment of 20 mg/kg CP and at 2 and 15 days after the first treatment of 5 mg/kg CP, respectively. These increases decreased to the levels in vehicle control rats at 1 or 2 weeks after the final dosing; thus micronucleus induction was a transient response (Fig. 3B).

4-NQO: Monitoring *Pig-a* mutant kinetics with two distinct approaches

Figure 4 shows the time course for *Pig-a* mutant frequency manifestation in rats administered single doses of 0, 50, 100, or 200 mg/kg 4-NQO. *Pig-a* mutant frequency was measured as both the frequency of CD45⁺/CD59⁻ RBCs and the frequency of HIS49⁺/CD59⁻ RBCs at Pre, 1, 2, 3, 4, 6, 8, and 10 weeks. Because of severe toxicity, the animals in the 200 mg/kg treatment group were euthanized 1 week after the treatment. Compared with the spontaneous *Pig-a* mutant frequencies, clear time- and dose-related increases in mutant frequency were detected in the treated rats, until a plateau was reached approx. 6 weeks after the 4-NQO treatments. The elevated mutant frequencies persisted for the remainder of the 10 week observation period. The kinetics of mutant manifestation and the induced mutant frequencies were similar using both staining protocols (Fig. 4A, 4B).

EMS: Monitoring of *Pig-a* mutant kinetics in HIS49-positive RBCs

We performed two experiments using the HIS49/CD59 method to monitor *Pig-a* mutant kinetics in rats treated with EMS. Figure 5 shows the results of Experiment 1 in which rats were treated with four weekly doses of 0 or 90 mg/kg EMS or a single dose of 360 mg/kg EMS. *Pig-a* mutant frequency was measured as the frequency of HIS49⁺/CD59⁻ RBCs at Pre, 2, 4, 6, and 8 weeks after the first dosing. Mean spontaneous *Pig-a* mutant frequencies in vehicle control rats ranged from 3.9×10^{-6} to 7.4×10^{-6} (Fig. 5). The maximum response with the single dose of 360 mg/kg was 17.8×10^{-6} at 4 weeks after dosing, which was a significant increase in mutant frequency. As for the four weekly doses of 90 mg/kg (total 360 mg/kg), the maximum response was 13.9×10^{-6} at 6 weeks after the first dosing. While this response was not significantly increased relative to the control, the increases at the other

Fig. 3. Percent of CD71-positive RETs among total RBCs (A) and the kinetics of MN-RET% and the frequency of CD59-negative RBCs (B) in rats treated with cyclophosphamide (CP). All data are the mean $\pm$ S.D. of data from five animals in each group. Dark arrows indicate the time of treatment. Doses of CP were as follows: 0 mg/kg $\times$ 10 daily doses (open circles), 5 mg/kg $\times$ 10 daily doses (open triangles), and single dose of 20 mg/kg (solid triangles). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$, 0.01, or 0.001 levels (*, **, or ***, respectively).

sampling times were significant.

In Experiment 2, rats were dosed orally for 28 days with vehicle or with 12 or 20 mg/kg EMS, or with single treatments of 180 or 360 mg/kg EMS. *Pig-a* mutant frequency was measured as the frequency of HIS49⁺/CD59⁻ RBCs at Pre, 2, 4, 6, and 8 weeks after the first dosing. Mean spontaneous *Pig-a* mutant frequencies in pre-dosing rats ranged from 2.2×10^{-6} to 6.7×10^{-6} (Data not shown). All the individual *Pig-a* mutant frequencies are exhibited on scatter plots (Fig. 6). Mean spontaneous *Pig-*

Fig. 4. Frequencies of CD59-negative RBCs in rats treated with 4-nitroquinoline-1-oxide (4-NQO). Dark arrow indicates the time of treatment. Doses of 4-NQO were as follows: 0 mg/kg (open circles), 50 mg/kg (solid triangles), 100 mg/kg (open triangles), and 200 mg/kg (solid squares). (A): The frequency of CD59-negative RBCs using the CD45/CD59 protocol. (B): The frequency of CD59-negative RBCs using the HIS49/CD59 protocol. All data are the mean $\pm$ S.D. of data from six animals in each group, except the 200 mg/kg group was $n = 4$. Statistically significant differences from vehicle controls are indicated at the $p < 0.05$, 0.01, or 0.001 levels (*, **, or ***, respectively).

a mutant frequencies in the vehicle control group at 2, 4, 6, and 8 weeks after the first dosing were 2.2×10^{-6} , 3.3×10^{-6} , 0.0×10^{-6} , and 3.0×10^{-6} , respectively (Fig. 6A, 6B, 6C, and 6D). Consistent with the findings of Experiment 1, a single dose of 360 mg/kg EMS increased the mean *Pig-a* mutant frequencies to more than 10×10^{-6} at 4 and 6 weeks after dosing; a similar response was found for the repeat dose of 20 mg/kg EMS at 6 and 8 weeks after the first dosing.

Fig. 5. Frequencies of CD59-negative RBCs in rats treated with ethylmethanesulfonate (EMS) using the HIS49/CD59 method. Doses of EMS were as follows: 0 mg/kg $\times$ 4 weekly doses (open circles), 90 mg/kg $\times$ 4 weekly doses (solid triangles), and single dose of 360 mg/kg (solid circles). All data are the mean $\pm$ S.D. of data from six animals in each group. Statistically significant differences from vehicle controls are indicated at the $p < 0.05$, 0.01, or 0.001 levels (*, **, or ***, respectively).

Comparability and sensitivity of the *Pig-a* mutant response using the two assay methods

In order to confirm the comparability of the *Pig-a* mutant responses as measured by the CD45/CD59 method and the HIS49/CD59 method, we compared the frequency of CD59-negative RBCs from individual rats following single treatment of 40 mg/kg ENU, and measured using both methods. Frequencies of CD59-negative RBCs increased similarly with time and dose using both methods. A positive correlation was observed between the frequencies of CD45-/CD59- RBCs and the frequencies of HIS49+/CD59- RBCs within the same animals ($r^2 = 0.8$, Fig. 7). These data were collected simultaneously in the experiments conducted with 4-NQO as a positive control group ($n = 4$ /group), so that the blood-sampling schedule for the ENU-treated rats is the same as the schedule described in Table 1 for 4-NQO-treated rats.

We also compared our in-house historical background/vehicle control data (April 2008 to December 2011) for the frequencies of *Pig-a* mutation measured either as CD45-/CD59- RBCs or HIS49+/CD59- RBCs (Fig. 8). The mean background frequency using the CD45/CD59 method was 12.7×10^{-6} . 17.7% (48/271) of the mutant frequen-

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Fig. 6. Frequencies of CD59-negative RBCs in rats treated with a single dose (180 or 360 mg/kg) or 28 days of repeat dosing (12 or 20 mg/kg) with EMS. Mutant frequencies were analyzed using the HIS49/CD59 method. Figure 6(A), (B), (C), and (D) are the data from assays conducted at 2, 4, 6, and 8 weeks after the first dosing, respectively. All individual data are exhibited as scatter plots and a bar shows the mean of each group. Statistically significant differences from vehicle controls are indicated at the $p < 0.05$, or 0.01 levels (*, or **, respectively).

cies were less than 5×10^{-6} , and 56.1% (152/271) less than 10×10^{-6} . As for the HIS49/CD59 method, the mean background frequency was 5.5×10^{-6} . 60.1% (104/173) of the mutant frequencies were less than 5×10^{-6} , and 86.7% (150/173) less than 10×10^{-6} .

DISCUSSION

The objective of this study was to compare two variations of an anti-CD59-based *Pig-a* assay (the CD45/CD59 and HIS49/CD59 methods) and evaluate their potential as screening tools for *in vivo* mutation by monitoring the kinetics of *Pig-a* mutant frequency in rats treated with well-known genotoxic chemicals. In addition, the *Pig-a* mutant frequency response was compared to the

response of the most commonly used *in vivo* genotoxicity assay, the micronucleus assay. Then the comparability and assay sensitivity of *Pig-a* mutant frequency detected by the CD45/CD59 and HIS49/CD59 methods were compared.

The potent gene mutagen, ENU, was shown in several earlier studies to produce large increases in *Pig-a* mutant frequency in dosed rats (e.g., Cammerer *et al.*, 2011; Dertinger *et al.*, 2010; Miura *et al.*, 2009). In this study, we compared the kinetics of *Pig-a* mutant frequency and micronucleus induction in rats after four weekly doses of ENU. While *Pig-a* mutant frequency accumulated and persisted in a time- and dose-related manner for at least 8 weeks after the first treatment, micronucleus induction was maximum at 2 days and diminished to

Fig. 7. Comparison of two distinct *Pig-a* assay protocols for measuring the frequency of CD59-negative RBCs. Data were from individual rats at 1, 2, 3, 4, 6, 8, and 10 weeks following single treatments of 40 mg/kg ENU.

near control levels within 1 week after the each treatment. These results demonstrate that the manifestation kinetics and persistence of these two endpoints are very different. Previous *Pig-a* mutant manifestation studies indicate that ENU-induced *Pig-a* mutant RBCs in rats persist for at least 6 months after the first treatment with ENU, and that *Pig-a* mutant RBCs accumulate after repeat dosing in a near-additive fashion (Dertinger *et al.*, 2010; Phonethepswath *et al.*, 2010; Miura *et al.*, 2009). Micronucleus induction is usually short-lived after single doses, but pseudo-persistent responses can appear to occur after daily repeated doses (Hamada *et al.*, 2001a). Thus, it would be possible to evaluate both chromosomal aberration and gene mutation if the *Pig-a* assay and the micronucleus assay were integrated into 28-day toxicology studies. Recent reports support this possibility (Cammerer *et al.*, 2011; Dertinger *et al.*, 2010).

CP is an alkylating agent that is often used as a positive control for the micronucleus test (Hamada *et al.*, 2001b). Similar to the results of the ENU experiment, a transient increase in MN-RET% was found after each treatment with CP. However, the *Pig-a* mutant frequency response following CP treatment was extremely weak, and, with the exception of a sample evaluated 4 weeks after the first treatment with 5 mg/kg CP, no statistically significant increases in *Pig-a* mutant frequency were found in this present study. In addition, the one positive measurement occurred at the point when the *Pig-a* mutant

Fig. 8. Distributions of background *Pig-a* mutant frequencies measured using two distinct *Pig-a* assay methods. *Pig-a* mutant frequency was measured as the frequency of CD59-deficient RBCs. (A); background *Pig-a* mutant frequencies measured with the CD45/CD59 protocol. (B); background *Pig-a* mutant frequencies measured with the HIS49/CD59 protocol. All data were from individual rats treated with vehicles as part of assays conducted at Teijin Pharma from April 2008 to December 2011.

frequency in vehicle control was at its lowest in the study. Thus, the biological significance of this increase in CP-treated rats is questionable, Walker *et al.* (2004) reported that the *Hprt* mutant lymphocyte frequency 8 weeks after treating rats with 20 mg/kg CP was approx. 2- or 3-times higher than the frequency in control rats. These results suggest that the mutagenic potential of CP is not

strong, at least for genes on the X-chromosome like *Pig-a* or *Hprt*, and that a spontaneous *Pig-a* mutant frequency as low and as stable as practical may be necessary for detecting any mutagenicity induced by CP.

4-NQO forms stable quinoline monoadducts with purine bases through the enzymatic reduction of its nitro group (Kondo, 1977). A previous report described time- and dose-dependent increases of *Pig-a* mutant RBCs in rats treated with 4-NQO (Stankowski *et al.*, 2011). In the present study, we demonstrated that both the CD45/CD59 and HIS49/CD59 assay detected similar *Pig-a* mutant responses. The major difference between the assays is that the CD45/CD59 assay assumes CD45-negative events are erythrocytes, whereas the HIS49/CD59 assay more directly identifies erythroid cells using a fluorescent antibody. These 4-NQO experiments and the comparison of data shown in Fig. 7 support the conclusion that these protocols are comparable, at least in terms of detecting induced mutant responses.

Significant increases in *Pig-a* mutant frequency were found after single and 4 weekly doses of EMS. Although the accumulation and the persistence of *Pig-a* mutant frequency were very similar for both treatment schedules during the 8 week observation period, the maximum responses in the single dose group and the 4 weekly dose group were found at 4 weeks and 6 weeks, respectively, although in the latter case the increase was not significant. This difference was consistent with a time lag in the rate of increase due to the fractionated dosing. A single dose of 360 mg/kg EMS produced similar small increases in *Pig-a* mutant frequency in both Experiments 1 and 2. In addition, small increases in *Pig-a* mutant frequency were detected in the 28-day repeat dosing with 20 mg/kg EMS at 6 and 8 weeks after the first dosing. Because only three animals were used in our dosing groups, our data may have suffered from type II error and therefore should be treated with caution. However, Dobo *et al.* (2011), using another approach to measure *Pig-a* mutant frequency, also found that measurement of *Pig-a* mutant RBCs was a useful method for measuring mutation in rats treated with EMS. Recent studies show that EMS exhibits a threshold for induced genotoxicity (Gocke *et al.*, 2009; Muller *et al.*, 2009) and it has been observed that, given the relative ease with which mutant frequency data can be generated with the *Pig-a* assay, the assay has potential for examining the possibility of *in vivo* genotoxicity thresholds for other DNA-reactive compounds (Dobo *et al.*, 2011). Our results suggest that the methods we have developed to measure *Pig-a* mutant RBCs might be useful for developing detailed *in vivo* dose responses for genotoxic agents; however, more work is needed to estab-

lish the accuracy and reliability of data generated by these *Pig-a* assays.

There was a positive correlation between the frequencies of CD45/CD59 RBCs and the frequencies of HIS49/CD59 RBCs within same animals following single treatments with 40 mg/kg ENU (Fig. 7). The data suggest that both methods measure comparable frequencies of *Pig-a* mutant RBCs. Our in-house historical background data, however, indicated that the vehicle/negative control mutant frequencies measured with the HIS49/CD59 method were less than half those measured with the CD45/CD59 method (Fig. 8). It is not clear why this is the case; however, we can think of at least two potential explanations for the difference. First, the higher background frequencies with the CD45/CD59 method might be related to insufficient exclusion of CD45-negative cells in PB that were not RBCs and which have poor expression of CD59. Unlike the protocol that was used in the international *Pig-a* mutation assay trial (Dertinger *et al.*, 2011c), our approaches did not use a density gradient to deplete nucleated leukocytes during the processing of samples. It is possible that positive selection for RBCs using HIS49/CD59 might produce a sample more enriched for RBCs, and thus be better approach for measuring *Pig-a* mutant RBCs. Second, the combination of fluorescently conjugated antibodies might be responsible for the difference in background frequency. We used antibodies labeled with FITC and PECy5 or FITC and APC for the CD45/CD59 method and the HIS49/CD59 method, respectively. APC is excited by the 635 nm laser, while both FITC and PECy5 are excited with the 488 nm laser. The data for HIS49/CD59 method were therefore acquired with two different lasers, which may have made it possible to discriminate the fluorescence with higher accuracy. Our analysis of the *Pig-a* background mutant frequencies indicated that, for individual samples, mutant frequencies generated with the CD45/CD59 method were consistently higher than mutant frequencies generated using HIS49/CD59 method. Overall, the mean background mutant frequency generated with the HIS49/CD59 method was approx. 5×10^{-6} ; we therefore consider that the HIS49/CD59 method is the better tool for measuring *Pig-a* mutant RBCs, especially for compounds that have a weak mutagenic potential.

Further improvement to the sensitivity of the *Pig-a* mutant assay is possible through the assaying of more RBCs than were interrogated in this present study. Immunomagnetic methods for depletion of wild-type RBCs (Dertinger *et al.*, 2011b) or enrichment of RETs (Kimoto *et al.*, 2011a) show promise in this regard. Dertinger *et al.* developed a system for depletion of CD59-positive RBCs

via immunomagnetic column separation (Dertinger *et al.*, 2011b). Their technique for enumerating *Pig-a* mutant frequencies is capable of interrogating more than 3×10^6 RETs and 1×10^8 RBCs in a single sample. In addition, we have developed an assay for measuring *Pig-a* mutant cells in rat RETs, the PIGRET assay, that employs magnetic enrichment of CD71-positive RETs and enables us to interrogate more than 1×10^6 CD71/HIS49-positive RETs for *Pig-a* mutation by FCM.

In conclusion, we have demonstrated that the RBC *Pig-a* assay using anti-CD45 or HIS49 antibody was useful as a screening tool for evaluating *in vivo* mutagenicity. In particular, the protocol using positive selection for RBCs with HIS49 antibody might be better tool for detecting the mutagenic potential of chemicals due to its relatively low, stable background *Pig-a* mutant frequency. These results also suggest that the *Pig-a* assay might be integrated into standard *in vivo* toxicology assays. More work is needed to define a standard *Pig-a* assay protocol to efficiently evaluate the risk of *in vivo* mutagenicity.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Robert H. Heflich for his critical review of this manuscript.

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