single assay had to be selected, the in vitro micronucleus assay would be the most appropriate choice.

There was discussion of a number of important technical aspects to the in vitro micronucleus assay that need to be taken into consideration. For instance, avoiding the use of chamber slides and washing the cells using centrifugation steps prior to slide preparation will minimize the interference with micronucleus scoring caused by agglomerates that sediment out of solution, as noted by some investigators [e.g., Lindberg et al., 2009]. Cytochalasin B is often used in the in vitro micronucleus assay (to block cytokinesis and create binucleated cells), which may affect the uptake of the nanoparticles by the cells due to inhibition of filaments involved in endocytosis [Doak et al., 2009]. To address this, it is essential to allow a suitable exposure period to the NM in the absence of cytochalasin B. The preferred duration of the exposure period without cytochalasin B is presently not clear. If the exposure period without cytochalasin B were at least one cell cycle it would ensure that the NMs are present during mitosis. This is the most likely stage at which those NMs could come into direct contact with the DNA, as the nuclear membrane breaks down and the genetic material intermingles with all the cytoplasmic components [Scientific Committee on Consumer Products, 2007; Singh et al., 2009; Doak et al., 2009, 2012; Gonzalez et al., 2011]. On the other hand, if the particles exert an indirect genotoxic effect shortly after cellular uptake or are small enough to enter the interphase nucleus, prolongation of the period without cytochalasin B could reduce the detection of a genotoxic effect, as the cells would have time to divide before cytochalasin B is added.

Finally, a variety of cell types can be used in the in vitro micronucleus assay and there does not appear to be a significant advantage of one cell type over others, provided that the cell type is able to take up small particles. Monolayer cells can perhaps be washed more easily, but the actual exposure to NMs is more uncertain unless agglomeration occurs, in which case the particles may then settle on top of the cells, perhaps in a nonuniform manner. Exposure of NMs to suspension cells may be more uniform, but it is harder to centrifuge out the particles. At this point, for hazard identification and regulatory purposes it was recommended that standard cell types as recommended in the recent OECD In Vitro micronucleus guideline (OECD 487) be used. Investigation of the intracellular content for various NMs across a variety of cell types and exposure scenarios (long/short/with and without cytochalasin B) would be useful to address this issue. It is recognized however that NM genotoxicity does not always require internalization of the particles [Bhabra et al., 2009].

Mammalian Mutation Assays

Not all forms of DNA damage can be detected by any one test. Cytogenetic assays will quantify gross chromosomal damage which is a relatively large event. However, more subtle genetic changes can also arise in response to a genotoxin such as point mutations including base substitutions, frameshifts or small amplifications and deletions. Thus, it was suggested that mammalian mutation assays should also be considered in a test battery, particularly as there appear to be some NMs that are missed by the in vitro micronucleus assay [Doak et al., 2012], and especially if the bacterial mutagenicity assays are not informative or not performed.

Positive Controls

One question that has been raised in a number of publications is the appropriate positive control for NM assays [Singh et al., 2009; Doak et al., 2009; Gonzalez et al., 2011]. In fact, there appears to be some thinking that testing of NMs is not appropriate until NM positive controls can be identified. Since the purpose of a positive control is to ensure that the assay system can respond and the analysts are able to appropriately score the assay, it was concluded that standard positive control chemicals are appropriate for use at this point. In the future, other positive controls, or NM-specific controls may be identified.

In Vitro Only Test Strategy

Taking into consideration the emphasis in the scientific field on reducing animal use, including the March 2009 ban on in vivo genotoxicity testing for cosmetic ingredients (EU Cosmetics Directive to the EU 7th Amendment), REACH legislation, etc., we discussed the testing approach if only in vitro assays could be used. One might consider that a battery of in vitro tests alone would not appropriately reflect the in vivo situation, for instance when genotoxicity is induced as a secondary mechanism by inflammation [Driscoll et al., 1997; Greim and Norppa, 2010]. For this situation, it was felt that there were other tools to evaluate potential for inflammation in in vitro assays (e.g., cytokine release) that could be used to address this. However, there is presently not enough information to judge how well in vitro co-culture models or assays, e.g., release of inflammatory cytokines, can predict in vivo effects. In general, the details of the interplay between inflammation, genotoxicity, and carcinogenicity are not well understood. It is not adequately known which stages and mechanisms in the inflammatory process are critical for genotoxicity, how strong the inflammatory effect would need to be, and how long the inflammation should continue to result in genotoxic effects. Materials provoking strong inflammation at low doses can be expected to be more hazardous than those inducing milder effects at high doses, but prolonged inflammation even at a low level may also be important.

On the basis of the known difficulties in preparing test solutions of NMs that reflect human exposure for use in

in vitro and in vivo assays, we discussed whether there was value in considering an approach that if a physiologically relevant dosing solution of a nanoparticle could not be prepared for in vitro assays, in vivo tests alone be conducted. However, it was concluded that in vitro testing was still appropriate even in situations where the level of agglomeration of the nanoparticle may not be exactly representative of human exposure (often times the nature of the NMs in human exposures and in the body is not fully known). It was felt that there would still be enough relevance for in vitro assays to provide useful information for hazard identification. Many of the various mechanisms by which NMs may be genotoxic [Gonzalez et al., 2008, Sargent et al., 2009] are detectable in vitro. The genotoxicity of readily or partly soluble NMs may depend on the soluble form [Greim and Norppa, 2010]. Thus, it was concluded that in vitro tests are an important part of a test battery.

Important Gaps to Consider for Future Work

There are many gaps in our knowledge about NMs and covering them all was beyond the scope of discussion in the limited time frame for this meeting. A few key gaps were highlighted. A continuing problem is the lack of complete physico-chemical characterization of the NM being tested and how to express concentration metrics. Additionally, the lack of established NM carcinogens/NM noncarcinogens was seen as a key gap in the development and validation of genotoxicity testing approaches. It was concluded, however, that hazard identification of NMs using current tests was still important. Another area lacking is a coordinated effort on testing a range of NMs within a battery of standard in vitro and in vivo assays for direct comparative purposes, as opposed to studies that are typically conducted on specific NMs using individual assays, many of which are not standardized. It was felt that NMs programs like that organized by OECD may help in this regard. Most importantly, it was felt there was a need for studies to be published even when showing negative results, and for attention to physiological relevance of the concentrations tested.

Breakout Group 2: Standard In Vivo Assays: Assessment/Strategy Development

Question: How would in vivo assays fit into a testing scheme for genotoxicity assessment of NMs?

Outcome of discussion:

The conventional testing strategy today is to assess in vitro and in vivo genotoxicity in a battery of standard assays, which will usually include the in vivo micronucleus assay (OECD TG 474) in bone marrow or peripheral blood. For the time being, this is required for regulatory submissions. However, current data support the view that the standard in vivo micronucleus assay is not capable of capturing all relevant NM modes of action (MOA), as, for example, may be the case when nanoparticles accumulate in organs other than the hematopoietic system.

Genotoxicity in vivo might be associated with NM initiated site of contact effects, e.g., through indirect mechanisms like bursts of reactive oxygen species (ROS) caused by inflammation [Downs et al., 2012], or with a NM leaching product. It is therefore likely that the conventional in vivo testing schemes used today do not capture all potential effects of NMs. Despite these uncertainties with in vivo testing, some potential in vivo approaches are discussed below.

It was suggested that the testing paradigm be modified so that other in vivo mammalian tests would be conducted prior to conducting an in vivo genotoxicity test. The purpose of these tests would be to characterize the pharmacokinetics using different routes of administration, and to address internal exposure, target tissue concentration and toxicity, as well as the persistence of NMs in the body/organ/cell. On the basis of this information, which is expected to help determine an MOA, a decision would be made concerning the need for a genotoxicity test and how it would be designed or selected.

Another topic that was debated was the route of exposure. For particulate materials it appears that this is of even higher importance than for other chemicals and it was suggested that the exposure route relevant for the human exposure scenario should be used, if possible.

The relevant length of exposure was also discussed. Most tests are done with short-term exposures although the most concerning exposure for humans is chronic exposure. Although this is also true for the testing of chemicals, the notion that nanomaterial uptake and distribution will usually be slower than for chemicals points towards the need for longer exposure schemes for NMs. Thus, for testing NMs, an alternative approach might be more appropriate, i.e., a short-term repeat-dose rodent study (7- or 28-days) conducted first to determine fate and distribution of the NM. Targeted tissue-specific in vivo genotoxicity studies would be conducted only when the target tissue, NM accumulation, and histological or microscopic changes had been determined. It also would be important to know if ROS formation or inflammation is the primary MOA, with genotoxicity as a secondary effect.

Depending on the outcome of the initial rodent study, the standard in vivo micronucleus assay (OECD TG 474) might be insufficient to capture accumulated genetic damage of the NMs. Consideration should therefore be given to variants of the MNT that allow the determination of effects after repeat-dose exposures (like the 28d integrated MNT). In addition, assays should be considered that can detect DNA damage that has accumulated over time in different organs, like transgenic assays using rodent models such as MutaMouse, BigBlue®, or *Gpt-*

delta mice which can measure long-term mutational effects in neutral transgenes. An alternative option is to use, for example, the recently developed Pig-a assay (if the hematopoietic cells are targets for the NMs).

Another assay that seems well suited for detecting the genotoxicity of NMs is the modified Comet assay. This assay can provide mechanistic information when coupled with DNA repair enzymes (e.g., formamidopyrimidine-DNA glycosylase (FPG), endonuclease III (ENDOIII), and 8-oxoguanine DNA glycosylase (hOGG1)) that generate DNA strand breaks in the presence of specific oxidative DNA adducts. The Comet assay has been reported to detect DNA damage resulting from ROS formation [Collins, 2009], one of the main MOAs postulated for NMs.

It was also proposed during the discussion that a NMs-specific positive control would not be required. There is no known positive NMs control that would be suitable for genotoxicity testing. In general, positive controls for in vivo genotoxicity tests are not considered to be of great importance for the validity of the data, but used as evidence of assay performance.

Important Gaps for Future Work

It could be worthwhile to explore the suitability of more unconventional model organisms such as *Drosophila* or Zebra fish for assessment of the cytotoxic, genotoxic, or germ cell effects of NMs. However, there are no data that show the suitability of these systems, and it was argued that especially for inhaled particles these models do not mimic human systems.

Breakout Group 3: Integration of New Technologies: Which Ones and When Are They Needed?

Question: If we could start over and create a "strategy" for assessment of the genotoxicity of NMs, *what tests might be chosen and why?*

Outcome of discussion:

Several issues were discussed for which the standard assays may not be optimal, and thus there is a need for consideration of new or adapted technologies. This includes assays for measuring NMs uptake into cells/systems, distribution into target organs, and macromolecule (DNA or protein) binding. Uptake studies require either NMs which can be monitored with existing methods (ICP-MS) or auto-fluorescent particles without leakage for imaging. The same requirement for labelled particles or tracking mechanisms applies to understanding the distribution and disposition of NMs within a surrogate animal or human. It is not clear if older DNA-binding mechanisms (e.g., ^{32}P postlabeling) would suffice for NMs interactions. It is likely that NMs DNA-binding capacity would require new approaches (e.g., fluorescence for some NMs), the results of which might be critical to define primary genotoxicity of the NMs. The screening of protein binding capacity may be of great help to select adequate genotoxicity assays and to interpret the results; protein arrays are in development to establish specific NMs profiles.

Concerning in vitro tests, the group considered that bacterial cells are not adequate, and that the eukaryotic systems need adaptations to avoid undesired interference of NMs with some vital components of the assay (interaction with formamidopyrimidine-DNA glycosylase (fpg) for the Comet assay; Cytochalasin B timing for the in vitro micronucleus assay). Cell suspensions (e.g., as used in the mouse lymphoma assay, MLA) could be a problem because of poor contact with NMs. The identification of DNA interactions and DNA strand breaks was considered important. The micronucleus and Comet assays were discussed as useful in this context. New in vitro assays that might be considered include genomics and proteomics as related to detection of immune system effects, oxidative stress, inflammation and other pathways. These technologies also can be used to assess pathway interactions. The identification of biomarkers was also discussed.

Concerning in-vivo genotoxicity assays, they are considered critical for assessment of NMs since they allow the detection of inflammation and, therefore, secondary genotoxicity. The strategy for in vivo testing requires consideration of the route of exposure, NMs uptake kinetics and the involvement of target tissues. Many studies are based on inhalation, but exposure occurs *via* different routes of which oral exposure is of particular interest.

As far as positive controls are concerned, at the present time their choice should be guided by the necessity to prove the quality and reliability of the assay.

Other Question Discussed by Group 3: How Do We Address the Concern for Reactive Oxygen Species (ROS)?

NMs can produce ROS either directly or through interaction with cell membranes or mitochondria. Photoreactive NMs could generate ROS triggered by ultraviolet (UV) or other radiation. Current tests designed for the detection of ROS are probably not sensitive enough. Moreover, they can be problematic due to the interference of the NMs with some of the fluorophores used in those assays or because of autofluorescence of some NMs. As alternatives, Electron Spin Resonance and spin trapping might be better suited to assess the cellular production of free radicals. A key question concerns the differentiation of exogenous and endogenous ROS. Research in this field using reporter cell lines or chemical assays such as aldehyde production might be helpful.

How Can We Cover Potential Novel Mechanisms of Action of NMs?

Besides ROS production, novel mechanisms of action of the NMs are being identified, in particular remodelling

of cytoskeletal proteins (tubulins and actins), which might lead to changes in cellular trafficking, transmembrane transport, cytokinesis (binucleation and/or polyploidy), and chromosome non-dysjunction (aneuploidy) [Gonzales, 2010]. The in vitro/in vivo micronucleus tests detect both chromosome breakage and aneuploidy and, if combined with molecular probing (centromere fluorescence in-situ hybridization, FISH, or kinetochore staining), the two mechanisms can be distinguished. Additional molecular and/immunofluorescent detection of cellular organelles might be helpful for elucidating the mechanisms. "Omics" technologies might be used to elucidate novel mechanisms of action of NMs.

Are There "Gaps" That New Assays or New Technologies Could Fill With Regard to Assessment of NMs?

The major gaps relate to the characterization of NMs, their interaction with macromolecules and the interaction among response pathways induced by the NMs. Differentiating effects of substances in different physical states, e.g., amorphous versus crystalline, and with different sizes and surface areas, remains a considerable challenge. A lot of work remains to address these challenges and to validate new assays and approaches.

OUTCOME OF THE PLENARY SESSION—CONCLUSIONS

The chairs of the individual breakout groups presented an overview of the results of the group work to all participants of the workshop. During an open discussion among all participants the following points were made:

i. Need for Analytical Assessment of NMs Recognized

The premise under which the discussions have taken place was that any study will need to be supported with adequate physical and analytical data in order to understand how the NMs in question behave in the vehicle/system used.

ii. Current Genotoxicity Test Battery Needs Modification for NMs

- *Bacterial assays*: There was agreement among the workshop participants that, while it seems that the bacterial assays may provide limited value for testing NMs, it is too early to eliminate them from the test battery. The generation of Ames study data for a wider range of materials, ideally in a coordinated manner in parallel with other in vitro genotoxicity tests, was encouraged.
- *In vitro micronucleus assay*: The utility of the in vitro micronucleus assay was underlined as it is able to capture damage from ROS induced stress as well as potential direct interaction with the spindle apparatus that may lead to aneuploidy. It was also emphasized that the standard OECD protocol for the in vitro micronucleus assay may need to be modified to capture effects triggered by NMs.
- *In vitro mammalian cell mutation assays*: These assays should be considered in the test battery as well, especially if it can be demonstrated that the bacterial assays are not informative for NMs-induced damage. Also the micronucleus assay will likely not cover the full spectrum of potential genotoxic effects.
- *In vivo assays*: It was suggested that repeated dose toxicity studies could provide very valuable information about potential mode of action and target organs. This information then could be used to guide the performance of in vivo genotoxicity assays which would only be performed if tissue toxicity were observed. Alternatively, genotoxicity endpoints can be built into the RDT studies, which would also take care of a concern that came up during the workshop, i.e., whether the classical acute protocols of in vivo genotoxicity assays are suitable at all to capture genotoxic events triggered by NMs which may accumulate in certain tissues over time. The workshop participants also wanted to emphasize the utility of assays that allow easy access to a wide range of potential target organs, like e.g., the Comet assay and transgenic rodent mutation assays.
- *Test battery*: The workshop participants agreed that results from a battery of standard in vitro and in vivo genotoxicity assays may not be sufficient to adequately assess the genotoxic potential of engineered NMs. Modifications to test methods, tests chosen, and additional information may be needed beyond that obtained from the standard genotoxicity studies.

iii. Mechanistic Context for NMs Should be Addressed

It was acknowledged by the workshop participants that mechanisms may be at play for NMs that a standard genotoxicity battery may not be able to capture, or that may generate wrong conclusions (i.e., direct versus indirect genotoxic effects). Therefore it was recommended that additional tools be used which should help unravel potential modes and mechanisms of action. Tools that were suggested during the workshop include markers for generation of ROS, e.g., electron spin resonance techniques and spin trapping, and "omics" technologies that help elucidate the affected pathways which could be targeted to prove or discard a hypothesis for a specific NM.

iv. Research Needs Acknowledged

It is recognized that, while there is current knowledge supporting the above recommendations, more research will need to be done before a genotoxicity testing approach for NMs can be finalized, and that there may not be a "one size fits all" strategy that will work for this very diverse class of materials. The discussion from this workshop helps lay the foundation for future workgroups and international bodies expected to address the safety assessment of NMs.

APPENDIX A: LIST OF PARTICIPANTS BY BREAKOUT GROUP

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Interlaboratory trial of the rat *Pig-a* mutation assay using an erythroid marker HIS49 antibody

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Flow cytometry

ABSTRACT

The peripheral blood *Pig-a* assay has shown promise as a tool for evaluating *in vivo* mutagenicity. In this study five laboratories participated in a collaborative trial that evaluated the transferability and reproducibility of a rat *Pig-a* assay that uses a HIS49 antibody reacts with an antigen found on erythrocytes and erythroid progenitors. In preliminary work, flow cytometry methods were established that enabled all laboratories to detect CD59-negative erythrocyte frequencies (*Pig-a* mutant frequencies) of $<10 \times 10^{-6}$ in control rats. Four of the laboratories (the in-life labs) then treated male rats with a single oral dose of *N*-nitroso-*N*-ethylurea, 7,12-dimethylbenz[*a*]anthracene (DMBA), or 4-nitroquinoline-1-oxide (4NQO). Blood samples were collected up to 4 weeks after the treatments and analyzed by flow cytometry for the frequency of CD59-negative cells among total red blood cells (RBCs; RBC *Pig-a* assay). RBC *Pig-a* assays were conducted in the four in-life laboratories, plus a fifth laboratory that received blood samples from the other laboratories. In addition, three of the five laboratories performed a *Pig-a* assay on reticulocytes (RETs; PIGRET assay), using blood from the rats treated with DMBA and 4NQO. The four in-life laboratories detected consistent, time- and dose-related increases in RBC *Pig-a* mutant frequency (MF) for all three test articles. Furthermore, comparable results were obtained in the fifth laboratory that received blood samples from other laboratories. The three laboratories conducting the PIGRET assay also detected consistent, time- and dose-related increases in *Pig-a* MF, with the RET MFs increasing more rapidly with time than RBC MFs. These results indicate that rat *Pig-a* assays using a HIS49 antibody were transferable between laboratories and that data generated by the assays were reproducible. The findings also suggest that the PIGRET assay may detect the *in vivo* mutagenicity of test compounds earlier than the RBC *Pig-a* assay.

1. Introduction

An *in vivo* gene mutation assay using the phosphatidylinositol glycan, class A gene (*Pig-a* in rodents, *PIG-A* in humans) as an endogenous reporter (*Pig-a* assay) is currently anticipated as being useful in quantitatively assessing genotoxicological risk including a radiation [1–3]. *Pig-a* is necessary for the synthesis of glycosylphosphatidylinositol (GPI), which binds a variety of proteins expressed on the cell surface. In mammals, *Pig-a* is the only gene necessary for GPI synthesis that is located on the X chromosome. Thus, a single mutation in the *Pig-a* gene can result in a deficiency in GPI-anchored proteins on the cell surface and GPI deficiency is virtually equivalent to *Pig-a* mutation [4,5]. These features of the GPI anchor system are exploited in the design of the *Pig-a* gene mutation assay that measures loss of a GPI-anchored protein marker by flow cytometry as a rapid assay for *in vivo* gene mutation.

Recent *Pig-a* assay development has focused on rats, resulting in various approaches for measuring *Pig-a* mutant cells in rat peripheral red blood cells (RBCs), reticulocytes (RETs), bone marrow erythroids, and spleen T lymphocytes [6–11]. Assays using peripheral blood erythrocytes have several advantages that make it possible to measure *in vivo* somatic mutation in a high-throughput

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manner. One advantage is that toxicant-induced RBC *Pig-a* mutant frequencies (MFs) persist in rat peripheral blood for at least months and accumulate as a result of repeat dosing [12,13]. Another advantage is that the assay can be performed with only a small volume of peripheral blood. These attributes benefit the integration of the *Pig-a* assay into other toxicology studies, especially repeated-dose toxicology studies.

An international interlaboratory trial has evaluated the transferability, reproducibility, and relative sensitivity of the rat *Pig-a* assay [14]. In this trial, anti-CD59-PE and SYTO 13 dye were used to label leukocyte-depleted blood samples to identify GPI-deficient *Pig-a* mutant cells. This system makes it possible to distinguish RETs from RBCs and to evaluate *Pig-a* MF in both populations simultaneously. The results from the trial indicated that the *Pig-a* assay was highly transferable and reproducible, and that the *Pig-a* assay could be a useful tool for evaluating *in vivo* mutagenicity.

While the protocol using the SYTO 13/anti-CD59 PE with leukocyte depletion is a well-known approach for detecting *Pig-a* mutations, a protocol developed by Dobrovolsky et al. also may have value as a screening tool for evaluating *in vivo* mutagenicity [10]. In this procedure, an antibody against the rat erythroid marker, HIS49, is employed to positively identify RBCs and RETs. A subsequent study on the HIS49 *Pig-a* assay in rat RBCs (referred to here as the RBC *Pig-a* assay) confirmed its potential for detecting mutagenic chemicals [15]. In addition, a recently developed assay for measuring *Pig-a* mutant cells in rat RETs (the PIGRET assay), which employs magnetic enrichment of CD71-positive RETs, is capable of interrogating more than 1×10^6 CD71/HIS49-positive RETs for *Pig-a* mutation [16]. However, it remains unknown whether these approaches are technically transferable between laboratories.

Collaborative work on the *Pig-a* assay has been conducted in Japan as part of a project entitled 'Study on the development of an integrated system of toxicology assays that can evaluate multiple endpoints,' supported by the Japan Health Sciences Foundation, Grants Nos. KHB1006 and KHB1209. The objective of the Grant KHB 1006 project is to develop a protocol that can acquire *in vivo* genetic toxicity data in a manner consistent with the '3Rs.' To this end, the *Pig-a* assay group has evaluated the transferability of the rat *Pig-a* assay using the HIS49 and CD59 antibodies. First, rat RBC *Pig-a* assay procedures developed by Teijin Pharma Limited (Teijin) were shared among four other participating labs, which included laboratories with no prior experience with assaying *Pig-a* mutation. The collaborating laboratories defined a standardized gating rule to reduce variability in the measurement of spontaneous CD59-negative cells (*Pig-a* mutants). They then confirmed the transferability of the protocol that was developed by measuring mutation in rats dosed with *N*-nitroso-*N*-ethylurea (ENU). In addition, they performed multilaboratory experiments using 4-nitroquinoline-1-oxide (4NQO) and 7,12-dimethylbenz[*a*]anthracene (DMBA) to evaluate interlaboratory portability and reproducibility. As part of the 4NQO and DMBA experiments, three of the five participants also conducted the PIGRET assay to test its transferability and reproducibility and to compare the responses in this assay with those of the RBC *Pig-a* assay.

2. Materials and methods

2.1. Reagents

ENU and DMBA were purchased from Sigma–Aldrich (St. Louis, MO, USA); 4NQO, olive oil and 0.5% (w/v) methylcellulose (400 cps) solution were purchased from Wako Pure Chemical Industry Co., Ltd. (Osaka, Japan). Anti-rat CD59 antibody (clone TH9, FITC-conjugated), anti-rat CD71 antibody (clone OX-26, PE-conjugated), anti-rat erythroid marker (clone HIS49, biotin-conjugated), and streptavidin-APC were purchased from BD Biosciences (Tokyo, Japan).

2.2. Animals

All animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals in each participant laboratory, and all experimental protocols were approved by the appropriate institutional review boards. Male CD (SD) rats were obtained from Charles River Laboratories (Kanagawa, Japan) (Table 1). The animals were given commercial feed and water *ad libitum* throughout the acclimation and experimental periods.

2.3. Administration of test agents and blood collection

After an acclimation period, the rats were administered a single dose of a test agent (ENU, 4NQO, or DMBA) by gavage. ENU was dissolved in warm (37 °C) PBS (pH adjusted to 6.0–6.1) at a concentration of 1.0 or 4.0 mg/mL (the ENU concentration was adjusted to account for moisture and stabilizer in the commercial preparation). 4NQO was suspended in 0.5% (w/v) methylcellulose solution at a concentration of 2.5, 5.0, or 10 mg/mL. DMBA was dissolved in olive oil at a concentration of 2.0, 4.0, or 6.0 mg/mL.

The description for study design is shown in Table 3. In the ENU study, test rats were administered a single dose of 0 (PBS vehicle), 10, or 40 mg/kg ENU by gavage ($n = 3$/group). In 4NQO study, rats were administered a single dose of 0 (0.5%, w/v MC vehicle), 25, 50, or 100 mg/kg 4NQO by gavage ($n = 5$/group). In DMBA study, rats were treated with a single dose of 0 (olive oil vehicle), 20, 40, or 60 mg/kg DMBA by gavage ($n = 5$/group). All test agents and the vehicle control were administered in a volume of 10 mL/kg rat weight. An ENU 40 mg/kg dosing group was used as a positive control group in the 4NQO and DMBA experiments for a confirmation of reactivity in rats (data not shown). Blood was collected from the tail vein or abdominal aorta and was promptly mixed with K2-EDTA to prevent clotting.

2.4. Blood processing for the RBC Pig-a assay

The sample preparation and the gating strategy for the RBC *Pig-a* assay were described previously [15]. The blood samples for the rat RBC *Pig-a* assay were processed within 72 h after the blood sampling. If shipping was required, the blood samples were refrigerated during shipment. Three microliters of blood/EDTA mixture were suspended in 200 μL of phosphate-buffered saline (PBS), and the cells were labeled with 0.125 μg of biotinylated HIS49 antibody and 1 μg of FITC-conjugated anti-rat CD59. After incubation for 1 h in the dark at room temperature, the samples were washed with PBS, centrifuged for 5 min at 1680 × *g*, and resuspended in 200 μL of PBS. Then, 0.2 μg of APC-conjugated streptavidin were added, and the samples were incubated for 15 min in the dark at room temperature. The specimens then were centrifuged for 5 min at 1680 × *g* and resuspended in approximately 1 mL of PBS.

2.5. Blood processing for the PIGRET assay

The sample preparation and the gating strategy for the PIGRET assay were described previously [16]. The blood samples for the PIGRET assay were processed within 24 h after the blood sampling. Approximately 80–150 μL of blood/EDTA were mixed with 200 μL of PBS, and then the cell suspension was layered onto Lympholyte-Mammal (Cedarlane Laboratories; Burlington, Ontario, Canada) and centrifuged to separate the nucleated cells and platelets from the RBCs. The RBC fraction was washed and then gently mixed with 100 μL of PBS. The resulting suspension was placed in a new tube containing 1 μg of PE-conjugated anti-rat CD71 and incubated for 15 min on ice. After being washed with 2 mL of 1× IMag™ Buffer (BD Biosciences) and centrifuged (1680 × *g*, 5 min), the cells were mixed with 50 μL of BD IMag™ PE Particles Plus-DM (BD Biosciences) and incubated for 15 min in a refrigerator. The samples were enriched for CD71-positive cells by processing with a BD IMagnet™ magnetic stand (BD Biosciences) according to the manufacturer's instructions. The enriched samples were labeled with HIS49 and anti-CD59 antibodies as indicated for total RBC labeling, with the exception that the incubation time for labeling enriched RETs was half that for the total RBCs. The final volume of the cell suspension was 500 μL.

2.6. Flow cytometry

Flow cytometric analysis to enumerate CD59-negative RBCs and RETs was conducted using flow cytometers equipped with 488-nm blue and 635-nm red lasers. The types of flow cytometers and software for data acquisition/analysis used in this study are shown in Table 1. The gating strategy for enumerating CD59-negative cells has been described previously [15,16]. All gates for the detection of CD59-negative RBCs and RETs were set using unstained and single-stained samples before evaluating the experimental samples. Approximately 1 million HIS49-positive RBCs and 1 million HIS49- and CD71-positive RETs were analyzed for expression of CD59 on the cell surface.

2.7. Statistical analysis

All data were expressed as the frequency of CD59-negative RBCs or RETs per million cells. Dunnett's multiple comparison *t*-test (non-parametric, two-sided) was

Table 1
Information of the participating laboratories in the collaborative study.

Participant laboratories (abbreviation)	Animals used in the experiments	Instruments, Software
Teijin Pharma Limited (Teijin)	Crl: CD(SD); 7 weeks (male)	FACSCanto II, FACSDiva ver. 6.1 (FACSAria I, FACSDiva ver. 4.1.2)[a]
Daiichi Sankyo Co., Ltd. (Daiichi Sankyo)	Crl: CD(SD); 7 weeks (male)	FACSCanto, FACSDiva ver. 6.0
Kaken Pharmaceutical Co., Ltd. (Kaken)	Crl: CD(SD); 6 weeks (male)	FACSCallibur, Cell Quest ver. 3.3
Mitsubishi Tanabe Pharma Corporation (Mitsubishi Tanabe)	Crl: CD(SD); 6 weeks (male)	FACSCallibur, Cell Quest ver. 3.3 (FACSCallibur, Flow Jo ver. 7.2.2)[b]
National Institute of Health Sciences (NIHS)	Not used (NIHS received blood samples from other participant laboratories and processed them into the *Pig-a* assay.)	FACSCanto II, FACSDiva ver. 6.1

[a] For the ENU study shown in Figs. 2 and 3.
[b] For the transferability study shown in Table 2.

Table 2
The spontaneous frequency of CD59-negative peripheral red blood cells in rats.

Region setting rule for CD59 negative red blood cells	Animal No.	Spontaneous *Pig-a* mutant frequency ($\times 10^{-6}$)				
		Teijin	Daiichi Sankyo	Kaken	Mitsubishi Tanabe	NIHS
No rule	01	1	7	12	11.1	13
	02	1	8	8	18.4	16
	03	4	4	1	11.2	25
	04	–	–	–	16.5	–
	Mean	2.0	6.3	7.0	14.3	18.0
	S.D.	1.7	2.1	5.6	3.7	6.2
After the rule applied	01	1	–	6	1.8	7
	02	0	–	7	5.5	5
	03	4	–	1	0.0	10
	04	–	–	–	0.0	–
	Mean	1.7	–	4.7	1.8	7.3
	S.D.	2.1	–	3.1	2.6	2.5

used for comparisons between the vehicle control group and each of the treatment groups at each time point. The levels of significance were set as $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Transferability of the RBC Pig-a assay using the HIS49 antibody

Five laboratories took part in the collaborative study (Table 1). The protocol for the assay was transferred from the Teijin lab (Reference lab) to other participant labs, and then participants attempted the RBC *Pig-a* assay in untreated rats to establish a common gate setting for the appropriate measurement of CD59-negative cells. In the first trial, the means of the spontaneous frequency in 2 of the 5 laboratories were more than 10×10^{-6} (Table 2), which was considered excessively high. Following discussions, a rule was agreed to in which the height of the gate region for CD59-negative RBCs was set to capture $99.0 \pm 0.1\%$ of the cells in the flow cytometry standard that was stained only with HIS49 antibody (Fig. 1). This rule was determined based on the evidence that most of the cells in the flow cytometry standard were included in this region according to the density plot analysis (Fig. 1). All participants, with the exception of the Daiichi Sankyo lab, adjusted their gate settings according to the rule and reprocessed their flow cytometry acquisition data. After the rule was applied, the spontaneous MFs of all the participating labs were $<10 \times 10^{-6}$ (Table 2). This rule was used in all labs to analyze samples in all subsequent studies.

3.2. Interlaboratory study with ENU

The participating labs conducted an ENU study whose design is shown in Table 3. Test rats were administered a single dose of 0 (PBS vehicle), 10, or 40 mg/kg ENU by gavage ($n = 3$/group). Blood samples were collected before the treatment and at 2 and 4 weeks after the ENU treatment. The National Institute of Health Sciences (NIHS) lab evaluated blood samples sent from the Teijin lab and conducted the *Pig-a* assay within 3 days after the blood collection. Fig. 2 shows the *Pig-a* MFs detected in each laboratory. The *Pig-a* MFs produced by a single dose of 40 mg/kg increased over time in all participating labs. We further analyzed the results of the ENU study by combining the data generated by the individual labs. Significant increases in *Pig-a* MF were produced by 10 mg/kg and 40 mg/kg ENU at both 2 and 4 weeks after the treatment (Fig. 3b and c). The *Pig-a* MF in the 10 mg/kg ENU group at 2 and 4 weeks after the treatment was 2.0- and 3.3-fold higher than in the control group, respectively. The *Pig-a* MF in the 40 mg/kg ENU group at 2 and 4 weeks after the treatment was 7.5- and 16.7-fold higher than in the control group, respectively. The *Pig-a* MF in the vehicle control group was stable and averaged less than 10 per million ($4.6–6.1 \times 10^{-6}$) (Fig. 3).

3.3. Interlaboratory study with 4NQO

The design for the 4NQO study is shown in Table 3. Four laboratories administered a single dose of 0 (0.5%, w/v MC vehicle), 25, 50, or 100 mg/kg 4NQO by gavage ($n = 5$/group). Three laboratories collected blood samples from the treated rats before and at 1, 2, and 4 weeks after the 4NQO treatment; one laboratory (Kaken) collected blood only at 2 and 4 weeks after dosing. All laboratories conducted the rat RBC *Pig-a* assay. The NIHS lab analyzed blood samples collected at the other four laboratories in an effort to evaluate data variability between the laboratories.

The results of multilaboratory experiments using 4NQO are shown in Fig. 4. All the in-life laboratories observed clear time- and dose-related increases in *Pig-a* MF after the treatment; for the high dose, the increases were statistically significant by 1 or 2 weeks

Table 3
Summary of experiments measuring *Pig-a* mutant cells in rats treated with genotoxic compounds.

Laboratories	Test compounds[a]	Dose (mg/kg)	Blood collection after the treatment (week)	Conducted assay
Teijin	ENU[b]	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO[c]	0, 25, 50, and 100	Pre, 1, 2 and 4	PIGRET assay
	DMBA[d]	0, 20, and 60	Pre, 1, 2 and 4	
Daiichi Sankyo	ENU	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO	0, 50, and 100	Pre, 1, 2 and 4	PIGRET assay
	DMBA	0, 20, and 60	Pre, 1, 2 and 4	
Kaken	ENU	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO	0, 25, 50, and 100	2 and 4	PIGRET assay
	DMBA	0, 20, 40, and 60	2 and 4	
Mitsubishi Tanabe	ENU	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO	0, 25, 50, and 100	Pre, 1, 2 and 4	
	DMBA	0, 20, and 60	Pre, 1, 2 and 4	
NIHS	ENU	Not conducted	Not conducted	*Pig-a* assay in total RBCs[e]
	4NQO			
	DMBA			

[a] The route of administration was gavage.
[b] *N*-nitroso-*N*-ethylurea.
[c] 4-Nitroquinoline-1-oxide.
[d] 7,12-Dimethylbenz[*a*]anthracene.
[e] NIHS analyzed blood samples delivered from other laboratories.

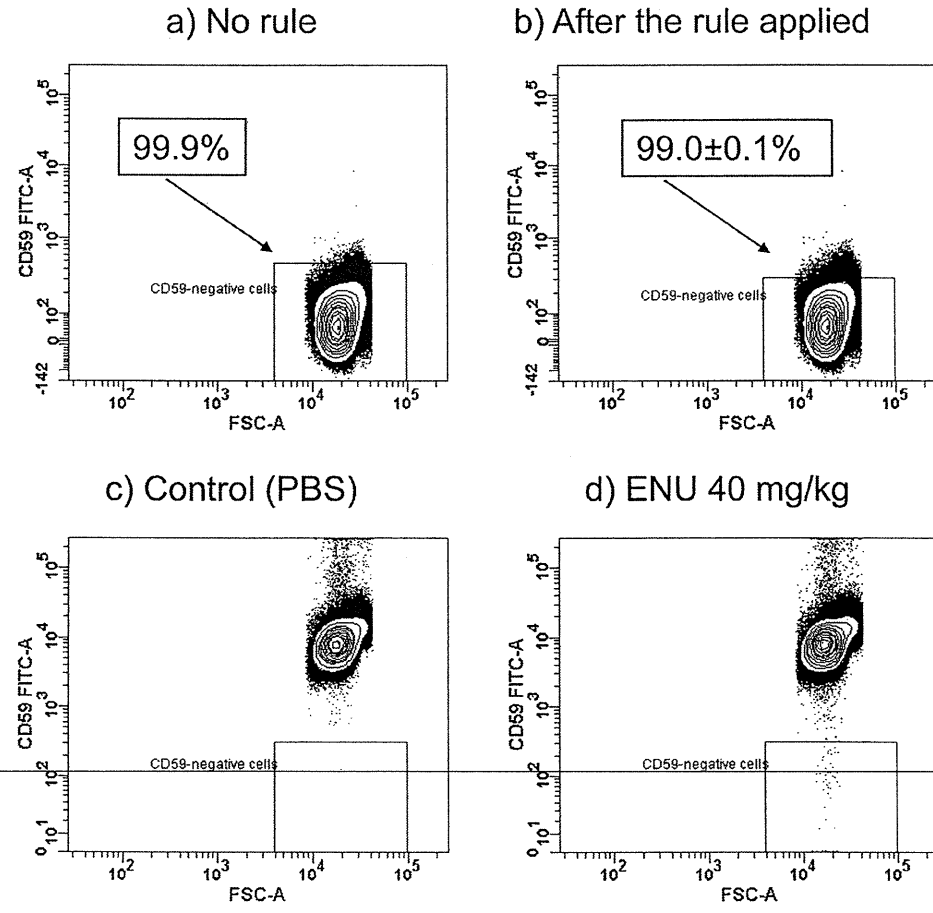

Fig. 1. Gating strategy for measuring CD59-negative cells. The red blood cells were defined by light scatter, and the positive expression of rat erythroid marker reacted with HIS49 antibody. (a) Plot data for pre-adjustment of the gate region using an HIS49 single-stained specimen. Most of the cells (99.9%) mimicked CD59-negative cells without anti-CD59 antibody. (b) Gate region setting after the rule was applied. The same specimen was used for the analysis in both (a) and (b). The height of the gate region for CD59-negative cells was set to include 99.0 ± 0.1% of cells. (c) and (d) Typical cytograms for measuring CD59-negative cells from the vehicle control (PBS) or 40 mg/kg *N*-nitroso-*N*-ethylurea groups.

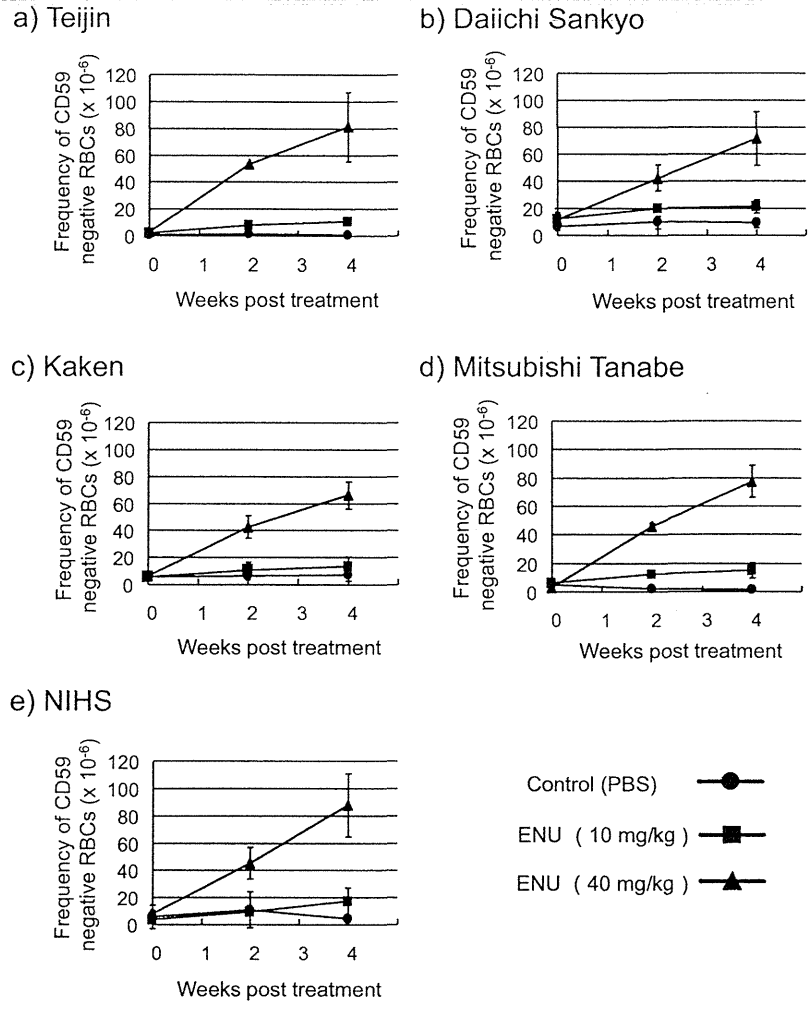

Fig. 2. *Pig-a* mutant frequencies measured by the participating laboratories in rats administered *N*-nitroso-*N*-ethylurea (ENU). The mutant frequencies were analyzed by enumerating the CD59-negative red blood cells (RBCs). Male SD rats were administered a single dose of ENU *via* gavage as shown in Table 3 ($n = 3$/group). Each graph shows the results by participating laboratory. The National Institute of Health Sciences (NIHS) received the blood samples from Teijin Pharma and conducted the RBC *Pig-a* assay within 3 days after the blood collection.

after the treatment (Fig. 4a, d, g, and j). Furthermore, comparable results were obtained at the NIHS lab, which analyzed the blood samples from the other participating labs (Fig. 4b, e, h, and k). The mean background *Pig-a* MFs in the vehicle control groups were generally less than 10 per million (64.3% of the samples had a MF less than 5.0×10^{-6}); two vehicle control means that were processed at the NIHS lab (pretreatment samples from the Teijin lab and 4-week post-treatment samples from the Kaken lab) were exceptions (Fig. 4b and h). Also, despite the uniformly low *Pig-a* MFs that were detected by the Mitsubishi Tanabe lab in their pretreatment samples (Fig. 4j), the NIHS lab detected non-significant increases in *Pig-a* MF in the same samples (Fig. 4k).

Fig. 4c, f, and i shows the results of PIGRET assays conducted on rats treated with 4NQO. Three of the five labs performed the PIGRET assay with the same blood samples used for the RBC *Pig-a* assay. The Teijin and Daiichi Sankyo labs produced comparable results, with apparent dose-related increases in the number of *Pig-a* mutant RETs by 1 week after the treatment (the Kaken lab performed the PIGRET assay only on 4 week samples). At 4 weeks after the treatment, all laboratories found significant increases in the 100 mg/kg dosing group. The mean RET *Pig-a* MFs in the vehicle controls were $0.0–1.8 \times 10^{-6}$, with all individual rat samples having MFs $<5.0 \times 10^{-6}$.

3.4. Interlaboratory study with DMBA

The design of the DMBA study is shown in Table 3. Four laboratories treated animals with a single dose of 0 (olive oil vehicle), 20, 40, or 60 mg/kg DMBA by gavage ($n = 5$/group). Similar to the 4NQO study, three laboratories collected blood samples from rats before the treatment and at 1, 2, and 4 weeks after dosing; the Kaken laboratory sampled blood only at 2 and 4 weeks after the dosing. All in-life laboratories conducted the rat RBC *Pig-a* assay, while the NIHS lab performed the RBC *Pig-a* assay on blood samples from three of these laboratories to evaluate data variability between the laboratories.

Fig. 5 shows the results of the multilaboratory experiments using DMBA. Clear time- and dose-related increases in *Pig-a* MF were observed in all laboratories, and the increases were statistically significant for the high-dose group by 1 or 2 weeks after the treatment (Fig. 5a, d, g, and j). Furthermore, comparable results were obtained at the NIHS lab, which analyzed the blood samples from other participating labs (Fig. 5b, e, and h). The mean *Pig-a* MFs in the vehicle control groups generally were stable and less than 10 per million (70.0% of the samples had a MF $<5.0 \times 10^{-6}$). The exceptions were the mean pretreatment sample from the Teijin lab and the 4 week vehicle control sample mean from the Teijin lab,

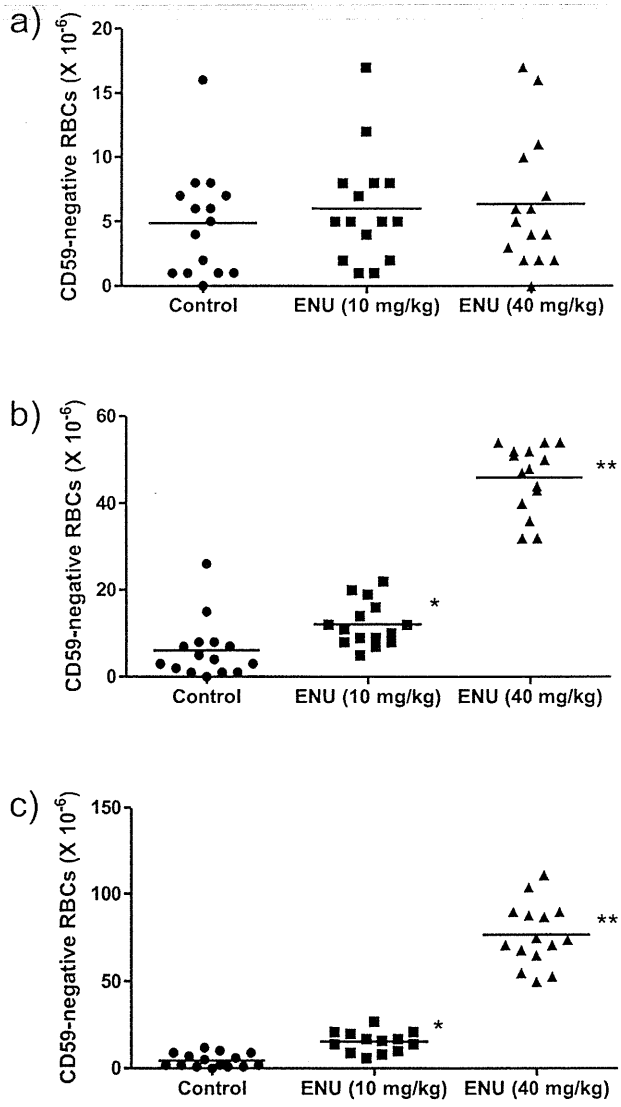

Fig. 3. Summary of *Pig-a* mutant frequencies in rats administered *N*-nitroso-*N*-ethylurea. The mutant frequencies were analyzed by enumerating CD59-negative red blood cells. (a), (b), and (c) display the combined data from assays conducted before and at 2 and 4 weeks after the first dosing, respectively. All individual data are displayed as scatter plots, and a bar indicates the mean of each group ($n = 13$ or 15). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$ or 0.01 levels (*, or **, respectively).

analyzed at NIHS (Fig. 5a and b). For their assays on pretreatment blood, the first sample analyzed by flow cytometry by the Teijin lab had a MF of 65×10^{-6}; however, no increases of *Pig-a* MF were found for the same rat when blood was analyzed at NIHS and at other sampling points at Teijin.

Fig. 5c, f, and i shows results from PIGRET assays conducted on blood from rats treated with DMBA. Three of the five labs conducted the PIGRET assay using the same blood samples used for the RBC *Pig-a* assay. Both the Teijin and Daiichi Sankyo labs found an apparent dose-related increase in the number of *Pig-a* mutant RETs by 1 week after the treatment. By 2 and 4 weeks after the treatment, all three laboratories found significant increases in the 60 mg/kg dosing group. The mean *Pig-a* MFs for RETs in the vehicle control rats from this study were $0.3–8.4 \times 10^{-6}$, with 79.6% of the samples having MFs $< 5.0 \times 10^{-6}$.

4. Discussion

Data from the five laboratories that participated in this collaborative study indicated that the protocol for the *Pig-a* assay using HIS49 antibody was transferable among different laboratories. First, a rule for setting flow cytometer gating was established that, in most cases, resulted in mean background MFs in the RBC *Pig-a* assay of $< 10 \times 10^{-6}$. This rule was applied to a gate in the FSC/CD59 cytograms for the purpose of accurately enumerating CD59-negative cells. After the rule was applied, more than 60% the *Pig-a* MFs in the vehicle control group were no greater than 5×10^{-6} (Table 2).

It was important that *Pig-a* mutants were identified consistently in the different labs and ideally that the background *Pig-a* MFs were reasonably consistent with previous measurements of background *Pig-a* MFs, and MFs of other X-linked reporter genes (like *Hprt*). A low spontaneous MF also was anticipated to benefit the assay in terms its power to detect the mutagenicity of test chemicals. With the HIS49 *Pig-a* assay, it was expected that the different laboratories could achieve equivalent assay power by adopting the rule. In fact, all laboratories demonstrated similar dose-dependent increases of *Pig-a* MF in the ENU experiment, the first experiment that was conducted after the rule was adopted (Fig. 2). The data that were collected (Fig. 3) indicated that the ENU treatment resulted in a consistent, statistically significant increase in *Pig-a* MF in each of the participant labs. This result indicates that the protocol was successfully transferred between participants, and that the participating labs had equivalent test power in the evaluation of ENU mutagenicity.

Based on the results of the ENU experiment, we then confirmed the reproducibility of the *Pig-a* assay between the participating laboratories by testing two other mutagenic agents, 4NQO and DMBA. Four of the participating labs carried out both the *Pig-a* assay and the animal experiment while the NIHS lab only carried out the RBC *Pig-a* assay, using blood samples shipped from other participating labs. The results indicated very small differences among the laboratories in terms of the RBC *Pig-a* MFs (Figs. 4 and 5). Both 4NQO and DMBA are known to increase the *Pig-a* MF in rats [13,15,17,18]. In this study, the participants found *Pig-a* MF responses that were similar to those in the previous studies. A recent report by Bhalli et al. demonstrated that both the RBC *Pig-a* assay and PIGRET assay produced similar results to the Litron's high throughput method which is the *Pig-a* mutant enrichment protocol in a Cis-platin study [19]. These results suggest that both the RBC *Pig-a* assay and PIGRET assay using HIS49 antibody are reproducible and sensitive methods for the detection of *in vivo* mutagenicity.

A second finding from this study was that the PIGRET assay can detect increases in *Pig-a* MF sooner after exposure than can the RBC *Pig-a* assay. In the 4NQO and DMBA experiments, 3 of the 5 participating labs also conducted the PIGRET assay, and most of their results indicated statistically significant increases in the treated animals (Figs. 4 and 5). It was notable that the PIGRET assay consistently detected increases in *Pig-a* MF at 1 week after the dosing, whereas the RBC *Pig-a* assay only occasionally detected an agent-induced increase at this sampling time. The PIGRET assay uses magnetic enrichment of RETs to increase the number of RETs that can be interrogated for mutations in a reasonable length of time [16]. The PIGRET assay not only has a good correlation with *Pig-a* MF measurements made in bone marrow erythroids, but it also has a low background *Pig-a* MF (usually $< 5 \times 10^{-6}$) [16], which benefits its ability to detect small increases in agent-induced MF. Although it is a challenging work, the PIGRET assay with a sorting instrument might be useful for a sequencing analysis of *Pig-a* gene because reticulocytes usually contain mRNA.

While the interlaboratory differences found for the HIS49 *Pig-a* assay were relatively small, it should be noted that some samples

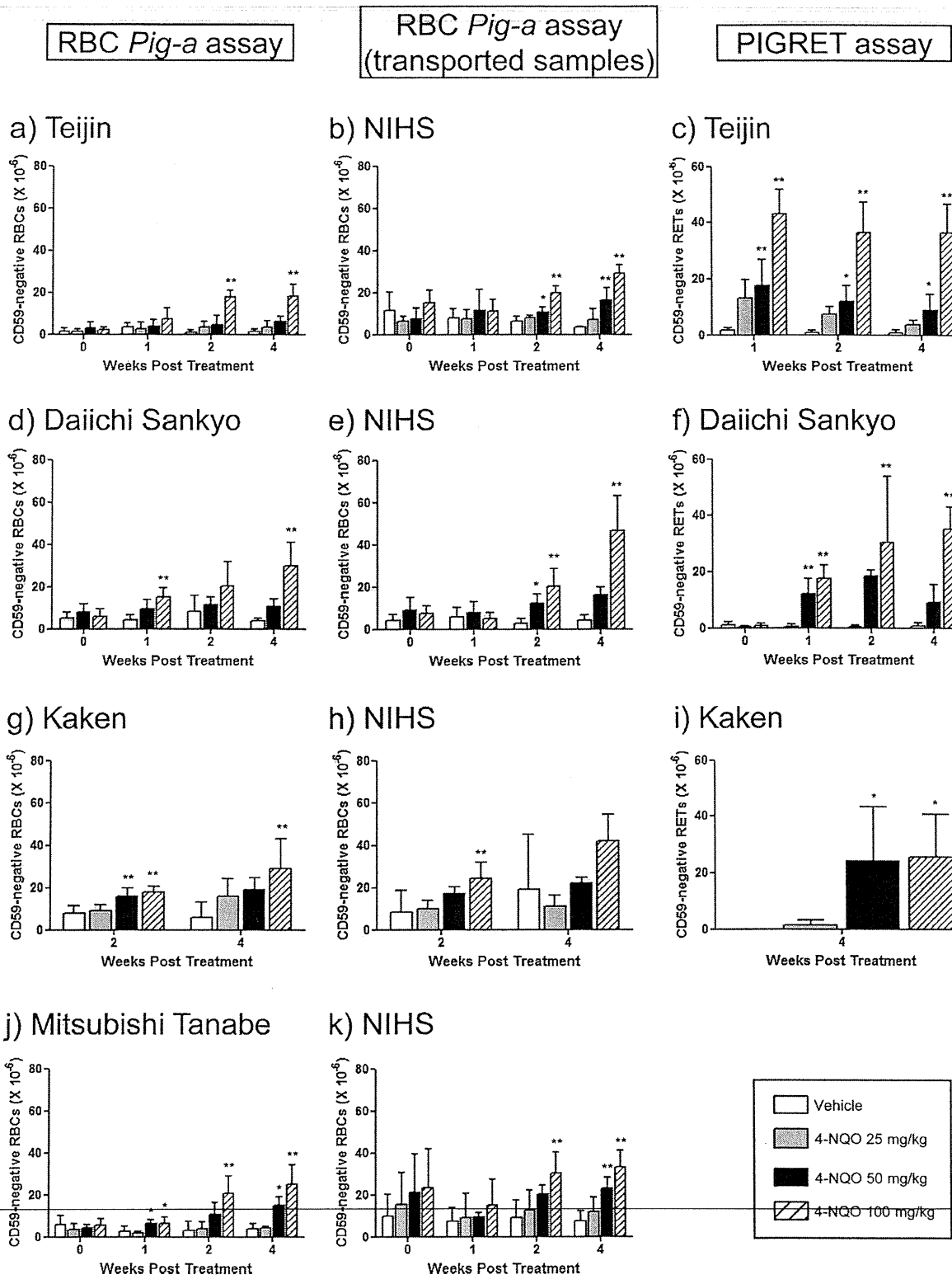

Fig. 4. *Pig-a* mutant frequencies in rats administered 4-nitroquinoline-1-oxide (4NQO). The mutant frequencies were analyzed by enumerating CD59-negative red blood cells (RBCs) or CD59-negative reticulocytes (RETs). Male SD rats were administered a single dose of 4NQO *via* gavage as shown in Table 3 ($n = 5$/group). Each graph shows the results by participating laboratory. The National Institute of Health Sciences (NIHS) analyzed blood samples from the other four laboratories using the RBC *Pig-a* assay ((b), (e), (h), and (k)). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$ or 0.01 levels (*, or **, respectively).

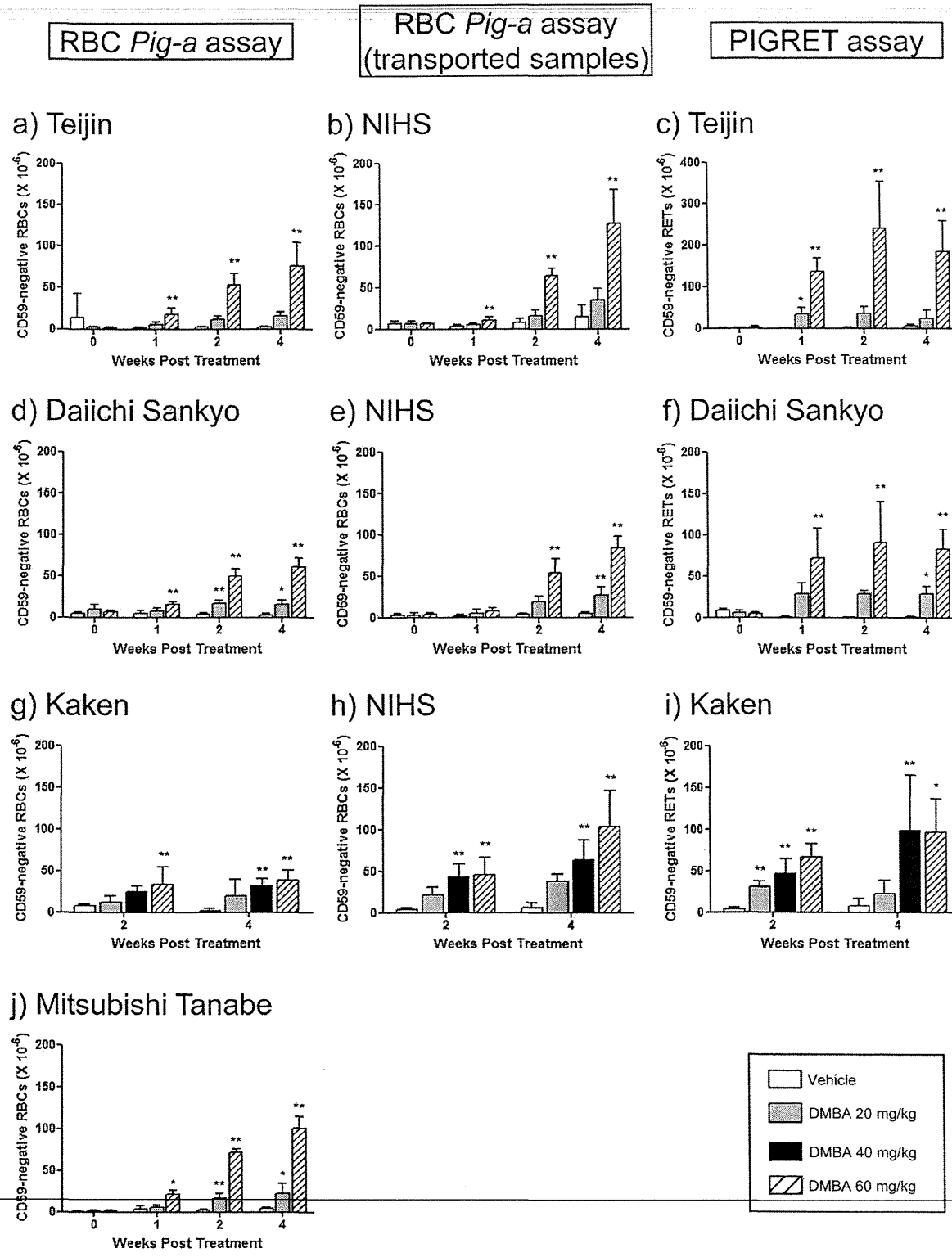

Fig. 5. *Pig-a* mutant frequencies in rats administered 7,12-dimethylbenz[*a*]anthracene (DMBA). The mutant frequencies were analyzed by enumerating CD59-negative red blood cells (RBCs) or CD59-negative reticulocytes (RETs). Male SD rats were administered a single dose of DMBA *via* gavage as shown in Table 3 ($n = 5$/group). Each graph shows the results by participating laboratory. The National Institute of Health Sciences (NIHS) analyzed the blood samples from the other three laboratories using the RBC *Pig-a* assay (Fig. 4(b), (e), and (h)). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$ or 0.01 levels (*, or **, respectively).

from the vehicle control groups had unusually high background frequencies. In particular, the RBC *Pig-a* MF of one of the pre-treatment samples from the Teijin lab was 65×10^{-6} (Fig. 5a). We speculate that this high value was caused by contamination from an unstained flow cytometry standard that was inadvertently left on a sample injection tube without cleaning. Other RBC *Pig-a* MF data from the same animal examined at 1–4 weeks after the dosing, and from this same blood sample at the NIHS lab, all had MFs of $<5 \times 10^{-6}$. Furthermore, some RBC *Pig-a* MFs measured by the NIHS lab indicated a high background frequency, although the other participating labs measuring MFs in these samples found that they had MFs of $<10 \times 10^{-6}$. These discrepancies are thought to be related to the shipment that these samples had undergone. It was noticed that some of these samples had dispersed within the tube, and portions of the blood dried. In addition, although it was exceedingly rare, some animals had consistently high spontaneous *Pig-a* MFs without dosing (data not shown). Therefore, it is important to clarify the way that outliers with a high spontaneous *Pig-a* MF are dealt with in designing and interpreting assays. As examples, a preliminary study might be conducted to identify animals with unusually high spontaneous *Pig-a* MFs so that they can be excluded from the experiment, or an experiment record sheet might be developed that identifies operational mistakes that could compromise the data. Also, further refinement of the protocol may ensure that the background *Pig-a* MF is consistently low.

Taken together, we found low variability in the *Pig-a* gene mutation data generated by the participating laboratories. In addition, in terms of measuring *in vivo* mutagenicity, the PIGRET assay, although technically more challenging than the RBC *Pig-a* assay, more consistently detected agent-induced increases in *Pig-a* MF at early sampling times than did the RBC *Pig-a* assay.

Conflict of interest statement

None.

Acknowledgement

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Environmental and Molecular Mutagenesis 54:747–754 (2013)

Research Article

Evaluation of *In Vivo* Genotoxicity Induced by *N*-Ethyl-*N*-nitrosourea, Benzo[*a*]pyrene, and 4-Nitroquinoline-1-oxide in the *Pig-a* and *gpt* Assays

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The recently developed *Pig-a* mutation assay is based on flow cytometric enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient red blood cells caused by a forward mutation in the *Pig-a* gene. Because the assay can be conducted in nontransgenic animals and the mutations accumulate with repeat dosing, we believe that the *Pig-a* assay could be integrated into repeat-dose toxicology studies and provides an alternative to transgenic rodent (TGR) mutation assays. The capacity and characteristics of the *Pig-a* assay relative to TGR mutation assays, however, are unclear. Here, using transgenic *gpt* delta mice, we compared the *in vivo* genotoxicity of single oral doses of *N*-ethyl-*N*-nitrosourea (ENU, 40 mg/kg), benzo[*a*]pyrene (BP, 100 and 200 mg/kg), and 4-nitroquinoline-1-oxide (4NQO, 50 mg/kg) in the *Pig-a* (peripheral blood) and *gpt* (bone marrow and liver) gene mutation assays. *Pig-a* assays were conducted at 2, 4, and 7 weeks after the treatment, while *gpt* assays were conducted on tissues collected at the 7-week terminal sacrifice. ENU increased both *Pig-a* and *gpt* mutant frequencies (MFs) at all sampling times, and BP increased MFs in both assays but the *Pig-a* MFs peaked at 2 weeks and then decreased. Although 4NQO increased *gpt* MFs in the liver, only weak, non-significant increases (two- or threefold above control) were detected in the bone marrow in both the *Pig-a* and the *gpt* assay. These findings suggest that further studies are needed to elucidate the kinetics of the *Pig-a* mutation assay in order to use it as an alternative to the TGR mutation assay. Environ. Mol. Mutagen. 54:747–754, 2013.

Key words: transgenic rodent mutation assays; glycosylphosphatidylinositol anchor; red blood cells; genotoxicity

INTRODUCTION

Since gene mutations are implicated in the etiology of cancer and other human diseases, *in vivo* genotoxicity tests are important as public health management tools. One such tool is the transgenic rodent (TGR) mutation assay, which quantitatively measures the accumulation of mutations in all organs, including germ cells [Nohmi et al., 2000]. The TGR mutation assay fulfills a need for a practical and widely available *in vivo* test for the assessment of gene mutation; the assay has been recommended by regulatory authorities for safety evaluations [COM, 2011; ICH, 2011] and international guidelines have been published describing the conduct of the assay [OECD488, 2011].

The recently developed *Pig-a* gene mutation assay is a powerful and potentially useful tool for evaluating *in vivo* genotoxicity that may complement the TGR assay [Miura et al., 2008a,b,2009]. Because the *Pig-a* gene is on the X-chromosome and involves the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, single mutations in the *Pig-a* gene can result in the loss of

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expression of GPI-anchored proteins, a phenotype that can be detected by flow cytometric evaluation with only a few μL of peripheral blood cells [Miura et al., 2009]. Additionally, *Pig-a* mutation appears to function in an apparently neutral manner, and the accumulated effects of repeat exposures can be evaluated. A standardized protocol for conducting and interpreting the assay, as well as the accuracy of the assay, however, have not been established, and the target organ for the assay currently is limited only to blood cells.

In this study, we performed the *Pig-a* assay and the *gpt* assay in the same animals and compared the performance of the two assays in detecting three known mutagens. We also developed a flow cytometric strategy for defining *Pig-a* mutant cells. This report describes the performance, effectiveness and advantages of the *Pig-a* assay in comparison with the *gpt* assay.

MATERIALS AND METHODS

Preparation of Chemicals

We dissolved *N*-ethyl-*N*-nitrosourea (ENU, Sigma-Aldrich Japan, Tokyo) in phosphate-buffered saline (PBS) (pH 6.0) at 10 mg/mL. Benzo[*a*]pyrene (BP, Wako Pure Chemical, Osaka) was suspended in olive oil at 10 mg/mL (for 100 mg/kg treatment) or 20 mg/mL (for 200 mg/kg treatment). 4-Nitroquinoline-1-oxide (4NQO, Sigma-Aldrich Japan, Tokyo) was suspended at 5 mg/mL in olive oil.

Antibodies

We obtained anti-mouse TER119 antibody for erythroid cell staining (clone TER-119, PE-Cy7-conjugated) and anti-mouse CD24 antibody (clone M1/69, FITC-conjugated) from BioLegend Japan (Tokyo).

Treatment of Mice

Animal experiments were conducted humanely according to the regulations of the Animal Care and Use Committee of the National Institute of Health Sciences (NIHS), Tokyo, and with their permission. *gpt* Delta C57BL/6J transgenic male mice were bred and maintained at the NIHS animal facility. They were housed individually under specific pathogen-free conditions with a 12-hr light–dark cycle and given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., Tokyo) *ad libitum*. At 8 weeks of age, five mice per group were given a single oral administration of ENU (40 mg/kg), BP (100 mg/kg or 200 mg/kg), 4NQO (50 mg/kg), or PBS (the negative control). Peripheral blood (18 μL) was withdrawn from a tail vein 2, 4, and 7 weeks after the treatments and used for the *Pig-a* assay. At 7 weeks, all mice were killed and bone marrow and liver samples were collected for the *gpt* assay.

Pig-a Mutation Assay

The *Pig-a* assay was performed with some modification of previously described methods [Miura et al., 2008a; Phonethepswath et al., 2008; Horibata et al., 2011; Kimoto et al., 2011]. Briefly, EDTA (dipotassium salt) was dissolved in distilled water to make a 12% solution and used as an anticoagulant. Peripheral blood (18 μL) was mixed with 2 μL EDTA solution. Two microliters of the blood/EDTA mixture was suspended in 0.2 mL PBS, and labeled with 1 μg each of anti-mouse TER119 and anti-mouse CD24 antibodies. The cells were incubated for 1 hr in the dark at room temperature, centrifuged (1000*g*, 5 min), resuspended in 2 mL PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences Japan, Tokyo). After gating for single cells, about 1×10^6 TER119-positive cells were analyzed for the presence of surface CD24, and the *Pig-a* mutant frequency (MF) was calculated as previously described [Horibata et al., 2011].

Gating Strategy for *Pig-a* Assay

Red blood cells (RBCs) were stained with anti-TER119, an antibody that specifically recognizes RBCs, and anti-CD24, an antibody used to detect GPI-anchored protein, as previously reported [Keller et al., 1999; Phonethepswath et al., 2008; Horibata et al., 2011; Kimoto et al., 2011]. Single cells, including RBCs and white blood cells (WBCs), were gated by light scatter (Fig. 1A). TER119-positive cells from this population (Fig. 1B) were analyzed further for the presence of the GPI-anchored CD24 antigen on the cell surface. The FITC-fluorescence intensities of RBCs without FITC-conjugated anti-CD24 were distributed as shown in Figure 1C, defining a gate that included 100% of lower FITC intensities of RBCs as "*Pig-a* mutant RBCs." This gating most likely included a number of events that were not true RBC *Pig-a* mutants, and therefore, to avoid artifactually inflating *Pig-a* MFs, we refined the gate for "*Pig-a* mutant RBCs" as the area encompassing a maximum of 99.0% of the lower RBC FITC staining intensities only (Figs. 1D and 1E).

gpt Mutation Assay

We extracted high molecular weight genomic DNA from liver and bone marrow cells using a Recover Ease DNA Isolation Kit (Agilent Technologies, Santa Clara, CA), rescued lambda EG10 phages using Transpack Packaging Extract (Agilent Technologies) and conducted the *gpt* mutation assay as previously described [Nohmi et al., 2000]. *gpt* MFs were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of colonies with rescued plasmids [Nohmi et al., 2000].

Statistical Analyses

The Kruskal–Wallis nonparametric test was used for comparisons among multiple groups at each time point. When significant differences were observed in the Kruskal–Wallis test, the Steel test was performed as a *post hoc* analysis for comparisons between the responses in the negative control and each treated group. Statistical analyses were performed using GraphPad Prism6 (GraphPad Software, La Jolla, CA) and Excel Statistics 2012 (Social Survey Research Information, Tokyo, Japan). For these analyses, a *P*-value of <0.05 was considered significant and two-tailed tests were performed.

Power analyses were performed using GraphPad StatMate2 software. GraphPad StatMate2 estimates the statistical power of detecting a difference between two sets of observations using an unpaired t-test (two-tailed). For these estimates, observations from our historical vehicle control data ($n = 95$, male C57BL/6 mice, 3–12 weeks of age, mean ± SD: 0.41 ± 0.91) were used to generate the power estimates (at the 80 and 95% level) of detecting true increases of 2-, 3-, etc., fold over the historical vehicle control MF at an $\alpha = 0.05$. SigmaPlot 12.5 also was used to estimate the effect of increasing the number of animals assayed on the power.

We also hypothesized that the power of the assay would be influenced by the number of erythrocytes interrogated for each sample, with the variability in the measurements, reflected in the SDs of mean *Pig-a* MFs for groups. In order to evaluate this effect, we determined assay power when the number of animals per group was 5 and the SDs of the mean control MF ranged from 0.1 to 15, comparing to the historical vehicle control ($n = 95$, SD = 0.91). Again, the historical vehicle control

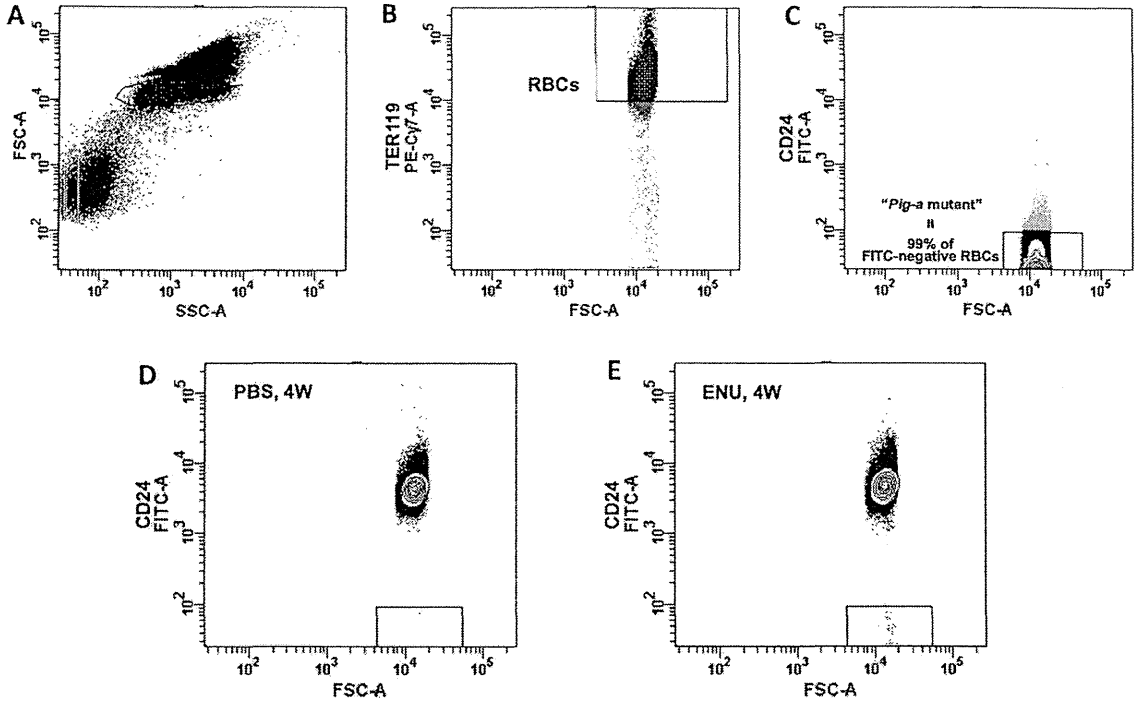

Fig. 1. Flow cytometeric analysis of mouse peripheral blood. A: Single cell populations were gated and further analyzed with anti-TER119 antibody. B: TER119-negative WBCs were excluded from the cell population gated in (A). TER119-positive RBCs were further analyzed with and without anti-CD24 antibody. C: TER119 positive cells were analyzed without anti-CD24 antibody staining so as to mimic *Pig-a* mutant RBCs. *Pig-a* mutant RBCs were defined by a gate encompassing at least 99% of lower intensities of FITC fluorescence associated with RBCs without anti-CD24 staining. **D,E**: Typical cytograms detecting *Pig-a* mutants. Approximately 1×10^6 TER119-positive cells derived from PBS- (D) or ENU- (E) treated mice were analyzed for CD24 expression.

MF (0.41×10^{-6}) was used as the control MF: minimal detectable fold-increases over vehicle control were calculated by dividing each estimated minimal detectable increase by the mean value of our historical vehicle control MF.

RESULTS

Pig-a Assay

The *Pig-a* mutant (CD24-negative) RBC population identified by flow cytometry after PBS or ENU treatment is shown in Figure 1D and 1E. MFs in the ENU-treated mice were significantly increased and the increase was modestly dependent upon time (mean ± SD for the PBS: 2 weeks after treatment, $0.80 \pm 0.45 \times 10^{-6}$; 4 weeks after treatment, $0.40 \pm 0.55 \times 10^{-6}$; and 7 weeks after treatment, $0.80 \pm 1.30 \times 10^{-6}$; mean ± SD for mice treated with 40 mg/kg ENU: 2 weeks after treatment, $23.00 \pm 6.96 \times 10^{-6}$; 4 weeks after treatment, $29.40 \pm 6.11 \times 10^{-6}$; and 7 weeks after treatment, $29.60 \pm 13.58 \times 10^{-6}$) (Fig. 2A).

In the case of 4NQO-treated mice, the average frequencies were more than twofold greater than controls at each of the time points studied (mean ± SD for mice treated with 50 mg/kg 4NQO: 2 weeks after treatment, $2.60 \pm 4.72 \times 10^{-6}$; 4 weeks after treatment, $1.00 \pm 1.00 \times 10^{-6}$; and 7 weeks after treatment, $2.20 \pm 4.38 \times 10^{-6}$) (Fig. 2B). Although there were no significant differences between the control and treated mice, there was relatively large range for the *Pig-a* MFs in individual 4NQO-treated mice (Fig. 2B, e.g. minimum MF was 0×10^{-6} and maximum MF was 11×10^{-6}). These results prompted us to conduct power analyses. At the 2-, 4-, and 7-week sampling times, the power of the assay to detect a significant increase in *Pig-a* MF after 4NQO-treatment was only 12%, 18%, and 10%, respectively, whereas the statistical power of the assay to reject the null hypothesis (i.e. no difference between control and treatment groups) and detect a significant increase in MF in the ENU-treated group was >95–99% at each of the sampling points.

Significant increases in *Pig-a* MF were observed for both BP doses at 2 weeks after treatment (Figs. 2C and 2D). For the mice treated with 100 mg/kg BP, the frequencies diminished with time, and no increase was evident 7 weeks after treatment (mean ± SD for mice treated with 100 mg/kg BP: 2 weeks after treatment, $9.25 \pm 2.63 \times 10^{-6}$; 4 weeks after treatment, $3.00 \pm 2.16 \times 10^{-6}$; and 7 weeks after treatment, $0.50 \pm 1.00 \times 10^{-6}$; with

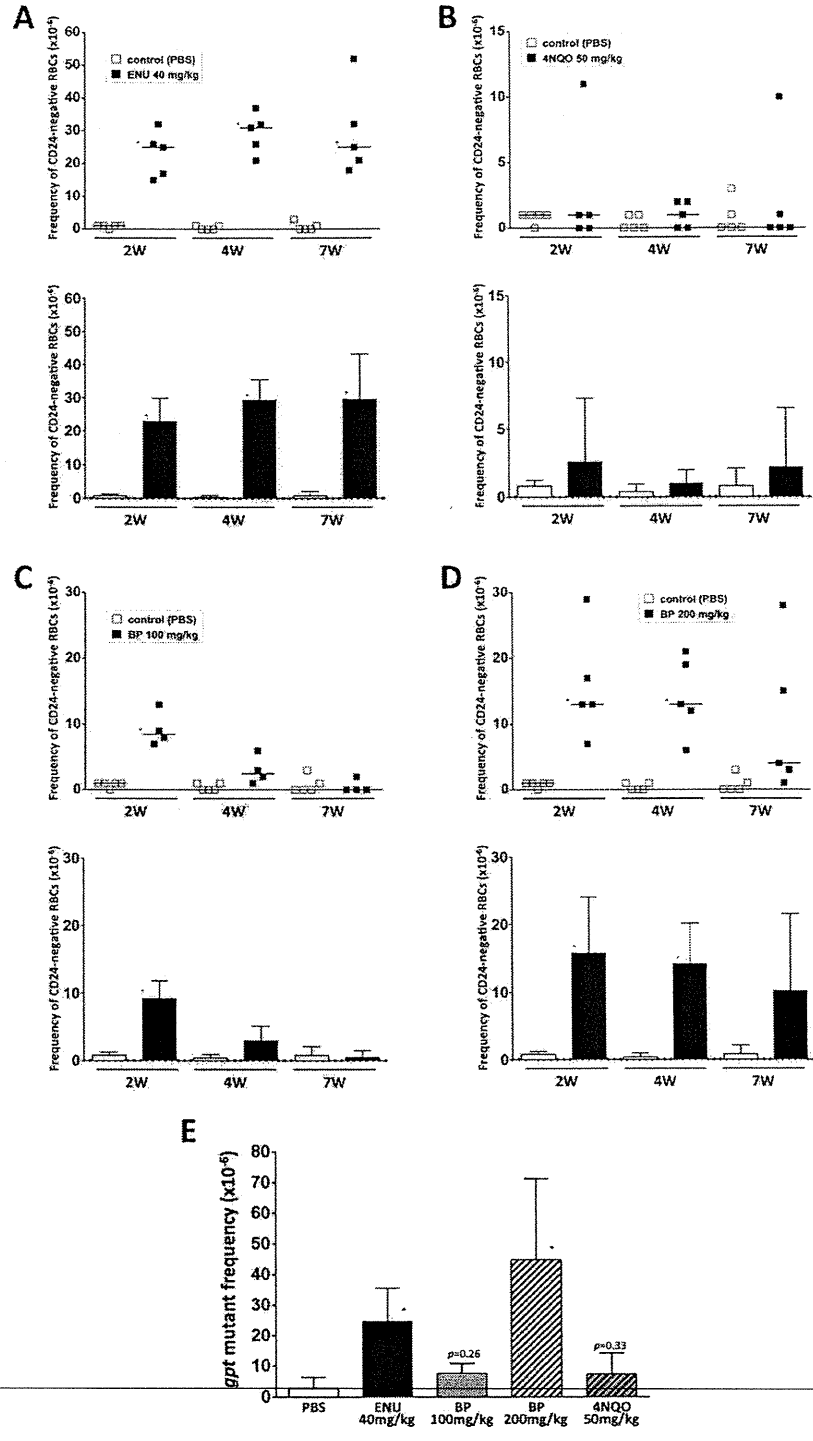

Fig. 2. Comparative analyses of *Pig-a* mutation in peripheral blood RBCs and *gpt* mutation in bone marrow cells. At 2, 4, and 7 weeks after treatment with (A) 40 mg/kg ENU, (B) 50 mg/kg 4NQO, (C) 100 mg/kg BP, or (D) 200 mg/kg BP, or PBS solvent, peripheral blood was withdrawn from the tail vein and analyzed by flow cytometry for the presence of CD24 on the surface of RBCs. Upper panels of (A)–(D): scatter plots with median bars. Lower panels of (A)–(D): the mean ± SD. (E) Seven weeks after treatment, all mice were killed and their bone marrow cells were isolated for the *gpt* assay. Frequencies are the mean ± SD of five animals per treatment group. $^{*}P < 0.05$.

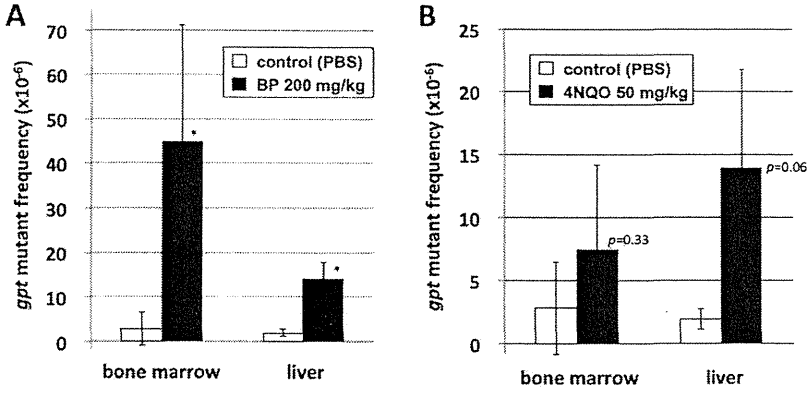

Fig. 3. Comparative analyses of *gpt* mutation in bone marrow and liver. Seven weeks after treatment, all mice were killed and liver samples were collected and analyzed by the *gpt* assay (bone marrow data are the same as in Fig. 2). The frequencies are the mean ± SD of data from five animals. $*P < 0.05$.

power analyses indicating that the assay had probabilities of >99%, >60%, and <10% of rejecting the null hypothesis, respectively, i.e. to distinguish between the negative control and BP-treated group). Although significant increases were measured at all sampling times, a similar reduction with time was detected in the mice treated with 200 mg/kg BP (mean ± SD for mice treated with BP: 2 weeks after treatment, $15.80 \pm 8.20 \times 10^{-6}$; 4 weeks after treatment, $14.20 \pm 5.97 \times 10^{-6}$; and 7 weeks after treatment, $10.20 \pm 11.34 \times 10^{-6}$; with power analysis indicating that the assay had probabilities of >95%, >99%, and >30%, respectively, to distinguish between the treated and control groups) (Figs. 2C and 2D).

gpt Assay on Bone Marrow and Liver

Compared with the solvent control animals (MF for PBS control group, $2.83 \pm 3.68 \times 10^{-6}$), significant increases in bone marrow *gpt* MFs were observed in ENU- and high-dose BP-treated mice (ENU, $24.70 \pm 10.49 \times 10^{-6}$; BP 200 mg/kg, $44.86 \pm 26.37 \times 10^{-6}$) (Fig. 2E), but not in low-dose BP- or in 4NQO-treated mice (100 mg/kg BP, $7.64 \pm 3.12 \times 10^{-6}$; 4NQO, $7.45 \pm 6.75 \times 10^{-6}$) (Figs. 2B and 2C). *gpt* MFs also were increased in the liver of high-dose BP- and 4NQO-treated mice (MF for PBS control group, $1.97 \pm 0.83 \times 10^{-6}$; 200 mg/kg BP, $14.04 \pm 3.76 \times 10^{-6}$; 4NQO, $13.92 \pm 7.83 \times 10^{-6}$) (Fig. 3).

DISCUSSION

TGR mutation assays, such as the *gpt* gene mutation assay, are an established method for monitoring *in vivo* genotoxicity in multiple tissues concurrently [for review, see Nohmi et al., 2000; Lambert et al., 2005; OECD488, 2011]. While the *Pig-a* gene mutation assay analyzes only one type of cells, i.e., blood cells, it has the advantage of not requiring the use of transgenic animals [Miura et al., 2008a,b]. The *Pig-a* assay has been undergoing extensive development and validation studies are presently being conducted [see the special issue of *Environmental and Molecular Mutagenesis* 52, 2011]. Based on the recent studies, it has been suggested that the *Pig-a* assay could be integrated into repeat-dose toxicology studies. The majority of *Pig-a* work to date has been performed with rats, with very little data being generated in mice, and this data gap needs to be addressed. Therefore, in this study, we analyzed both transgene and *Pig-a* mutation using *gpt*-delta transgenic mice.

Our results using a single oral administration of ENU were consistent with those of previously reported mouse studies [Bhalli et al., 2011a; Horibata et al., 2011; Kimoto et al., 2011]. The ENU-induced MFs detected by the *Pig-a* and *gpt* assays were similar, suggesting that both assays were able to detect ENU genotoxicity equally well.

We also detected dose-dependent increases of *Pig-a* MF in BP-treated mice (Figs. 2C and 2D). The MFs in the mice treated with both low- and high- doses of BP peaked at 2 weeks after treatment and declined thereafter. These time-dependent reductions in *Pig-a* MF differed from the kinetics of ENU–induced *Pig-a* mutation. Phonethepswath et al. reported on the kinetics of *Pig-a* mutation in RBCs from Wister rats treated with ENU, 7,12-dimethyl-1,2-benz[*a*]anthracene, N-methyl-N-nitrosourea, 4NQO and BP [Phonethepswath et al., 2010]. While erythroid progenitors normally mature over a period of 7–14 days *in vitro* [Iscove and Sieber, 1975], it takes 4–6 months to re-establish stable hematopoietic stem cell numbers following bone marrow transplantation [Jordan and Lemischka, 1990]. These observations imply that ENU may effectively mutate hematopoietic stem cells, and that a large proportion of *Pig-a* mutants in BP-treated mice may be due to mutations induced in erythroid progenitors.