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Erythrocyte-based *Pig-a* gene mutation assay: Demonstration of cross-species potential

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Abstract

Glycosylphosphatidylinositol (GPI) anchors attach specific proteins to the cell surface of hematopoietic cells. Of the genes required to form GPI anchors, only *Pig-a* is located on the X-chromosome. Prior work with rats suggests that the GPI anchor deficient phenotype is a reliable indicator of *Pig-a* mutation [Bryce *et al.*, *Environ. Mol. Mutagen.*, 49 (2008) 256–264]. The current report extends this line of investigation by describing simplified blood handling procedures, and by testing the assay principle in a second species, *Mus musculus*. With this method, erythrocytes are isolated, incubated with anti-CD24-PE, and stained with SYTO 13. Flow cytometric analyses quantify GPI anchor-deficient erythrocytes and reticulocytes. After reconstruction experiments with mutant-mimicking cells demonstrated that the analytical performance of the method is high, CD-1 mice were treated on three occasions with 7,12-dimethyl-1,2-benz[a]anthracene (DMBA, 75 mg/kg/day) or ethyl-N-nitrosourea (ENU, 40 mg/kg/day). Two weeks after the final treatment, DMBA-treated mice were found to exhibit markedly elevated frequencies of GPI anchor deficient erythrocytes and reticulocytes. For the ENU experiment, blood specimens were collected at weekly intervals over a five week period. Whereas the frequencies of mutant reticulocytes were significantly elevated one week after the last administration, the erythrocyte population was unchanged until the second week. Thereafter, both populations exhibited persistently elevated frequencies for the duration of the experiment (mean frequency at termination = 310×10^{-6} and 523×10^{-6} for erythrocyte and reticulocyte populations, respectively). These data provide evidence that *Pig-a* mutation does not convey an appreciable positive or negative cell survival advantage to affected erythroid progenitors, although they do suggest that affected erythrocytes have a reduced lifespan in circulation. Collectively, accumulated data support the hypothesis that flow cytometric enumeration of GPI anchor deficient erythrocytes and/or reticulocytes represents an effective *in vivo* mutation assay that is applicable across species of toxicological interest.

Key terms

Pig-a; mutation; GPI anchor; flow cytometry; CD24; erythrocytes

1. Introduction

This laboratory has described a flow cytometry-based approach for quantifying *in vivo* mutation [1]. The method is based on *Pig-a*, a gene whose product is essential for the synthesis of glycosylphosphatidylinositol (GPI) anchors [2]. Hematopoietic cells require GPI anchors

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to attach a host of proteins to their cell surface, for instance CD24, CD59, CD55, and CD48 [3]. Importantly, of the genes required to form GPI anchors, only *Pig-a* is located on the X-chromosome. Together, this information has led us to hypothesize that GPI anchor deficiency should represent a reliable phenotypic marker of *Pig-a* mutation.

In addition to the proof-of-principle data reported by Bryce *et al.*, other important feasibility data have been generated by Miura and colleagues, who demonstrated that both rat blood erythrocytes and splenic lymphocytes can be readily evaluated for the GPI anchor deficient phenotype [4]. Furthermore, through DNA sequencing experiments, these investigators reported that cells that exhibit the GPI anchor deficient phenotype are in fact bona fide *Pig-a* mutants [5].

Beyond these rat-based investigations, a wealth of information regarding the underlying biology of GPI anchor deficiency has been developed through the study of a human disease caused by *Pig-a* mutation, Paroxysmal Nocturnal Hemoglobinuria (PNH). Investigations into PNH etiology have shown that the GPI anchor deficient phenotype is invariably due to *Pig-a* mutation [6], each important class of mutation can ablate GPI anchors (base-pair substitutions, frame-shifts, small and large insertions/deletions) [7–8], and that *Pig-a* mutation is neutral, meaning it does not convey a cell survival advantage or disadvantage [9–10]. Based on these characteristics, combined with their own extensive work in this field, Araten *et al.* [11] speculated “...quite apart from its relevance to the pathogenesis of PNH, the analysis of the *PIG-A* gene may serve for the detection and quantitation of somatic mutations in hematopoietic cells in general.”

When setting out to test the feasibility of a *Pig-a* based assay, our laboratory initially focused on rat erythrocytes using a 3-fluorochrome method. Two populations of erythrocytes were studied: circulating red blood cells without regard to maturity (RNA-negative as well as RNA-positive, abbreviated RBCs), and the immature fraction (RNA-positive, or reticulocytes, abbreviated RETs). The current investigation also focuses on these two populations, and was initiated in an effort to 1) reduce the technical requirements for conducting the assay by developing a 2-fluorochrome method, and 2) test the cross-species potential of the assay system. A detailed description of the simplified staining/analysis methods, as well as mouse data generated from 7,12-dimethyl-1,2-benz[a]anthracene and ethyl-N-nitrosourea treatments, are reported herein.

2. Materials and Methods

2.1 Reagents

Sesame oil, ethyl-N-nitrosourea (ENU; cas no. 759-73-9), and 7,12-dimethyl-1,2-benz[a]anthracene (DMBA; cas no. 57-97-6) were purchased from Sigma-Aldrich, St. Louis, MO. Phosphate buffered saline (PBS; phenol red and Ca⁺⁺/Mg⁺⁺ free) was from Mediatech, Inc., Herndon, VA. A solution of 0.9% NaCl for injection was obtained from Ricca Chemical Co., Arlington, TX. Disodium-EDTA (cas no. 6381-92-6) was from JT-Baker, Phillipsburg, NJ. Purified unlabeled and PE-conjugated anti-mouse CD24 (clone number M1/69) were from BD Biosciences, San Jose, CA. SYTO 13 was obtained from Invitrogen, Carlsbad, California.

2.2 Animals, Treatments, Blood Draws

Female CD-1 mice were purchased from Charles River Laboratories, Wilmington, MA. Mice were allowed to acclimate for at least one week before treatment. Water and food were available *ad libitum* throughout the acclimation and experimental periods. Treatment with mutagens occurred three times at two day intervals when animals were between 7 and 8 weeks of age. ENU was administered at 40 mg/kg/day and DMBA at 75 mg/kg/day. All treatments occurred

via intraperitoneal injection. Solvents were 0.9% saline and sesame oil for ENU and DMBA, respectively, and vehicle exposed mice served as negative controls.

For the DMBA experiment, three mice were randomly selected for the 0 and 75 mg/kg/day groups. Blood collection occurred two weeks after the final administration. For the ENU experiment, twenty mice were treated with genotoxicant and blood was collected from four of these mice on each of five occasions: 1, 2, 3, 4 and 5 weeks after the final administration (i.e., no serial draws so that erythropoiesis function was not disturbed). Five mice were assigned to the vehicle control group, and for each of the five time points, one of these mice supplied a blood specimen.

Blood harvests occurred via heart puncture using a 26 gauge needle and syringe following CO₂ overdose. To prevent coagulation, the syringes contained approximately 90 µL of PBS with heparin at 500 units/mL. Approximately 500 µL of blood per mouse was obtained and stored on ice or in a refrigerator until staining occurred (same day). (Note that while 500 µL were collected per mouse, we estimate that only 30 to 50 µL were needed to provide 2×10^8 cells for staining, Section 2.3, below.)

2.3 Cell Staining

Each heparinized whole blood specimen was diluted 1:1 with PBS. One part diluted blood (typically 1000 µL) was then layered on top of eight parts room temperature Lympholyte Mammalian Cell Separation Reagent (CedarLane, Burlington, NC; cat. no. CL5110) in a 15 mL conical polypropylene tube. To enrich for erythrocytes, specimens were centrifuged at 800g for 20 minutes. The resulting supernatants were carefully aspirated and 1 mL of PBS was gently added to the side of each tube with the goal of minimally disturbing the erythrocyte pellets. The PBS rinse solution was removed and a second addition of PBS (1 mL/tube) was added as before. After removing the second rinse volume, a Pipetman was used to transfer each erythrocyte pellet to a new 15 mL polypropylene tube containing 10 mL PBS. The specimens were washed twice with 10 mL PBS, the first for 10 min and the second time for 5 min at 300g. Cells were finally resuspended with 5 mL PBS and placed on ice.

A Coulter Counter (model ZM) was used to determine the density of the fractionated, washed erythrocytes. 2×10^8 cells were then transferred to a tube containing 100 µL antibody solution (10 µL stock anti-CD24-PE for every 90 µL PBS plus 2% v/v fetal bovine serum). After incubating for 30 min on ice, cells were washed with 10 mL PBS per tube. After centrifugation and aspiration, pellets were resuspended with 1 mL SYTO 13 solution (156 nM, prepared in PBS). After incubating for 30 min at room temperature, samples were moved to ice until flow cytometric analysis occurred (within approximately 2 hrs).

2.4 Instrument Calibration Standards

The extreme rarity of *Pig-a* mutant cells represents a challenge to flow cytometer operators who ideally set PMT and compensation settings based on fluorescence profiles of both positive and negative events. This was addressed by generating mutant mimicking cells in parallel with the experimental samples each day that flow cytometric analysis occurred. The mutant-like cells consisted of RBCs that lacked anti-CD24-PE fluorescence. These specimens were generated from mouse blood that was fractionated and stained with SYTO 13 as described above. However, instead of being incubated with PE-conjugated anti-CD24, the same amount of unlabeled anti-CD24 was used. This substitution generated RBCs that exhibited the mutant (GPI-anchor deficient) phenotype. Before experimental samples were analyzed, equal volumes of these mutant-like cells were added to a tube that contained vehicle control RBCs that were stained according to the standard staining protocol. The resulting instrument calibration specimen provided sufficient numbers of RBCs that exhibited a full range of PE and SYTO

13 fluorescence intensities that were useful for optimizing PMT voltages and fluorescence compensation settings on a daily basis. The position of the CD24-negative cells also provided a rational approach for defining the vertical demarcation line that was used to distinguish CD24-negative from CD24-positive events.

2.5 Reconstruction Experiments

The analytical performance of the staining and data acquisition procedures were evaluated in several independent experiments by staining a naïve mouse blood specimen as described above, and then adding various, known numbers of mutant mimicking RBCs. As with the biological standards for instrument set up purposes, these cells exhibited a GPI-anchor deficient phenotype, and their frequencies were determined via flow cytometric analysis as described below.

2.6 Data Acquisition

A FACSCalibur providing 488 nm excitation and running CellQuest Pro v5.2 software was used for these studies (BD Biosciences). For analyses that sought to measure the frequency of CD24-negative RBCs, data acquisition was triggered on the forward scatter parameter, and the sample rate was set to low. This facilitated efficient collection of 1×10^6 RBCs per specimen (typically ≤ 5 minutes).

For analyses that sought to measure the frequency of RETs that were CD24-negative, the sample rate was medium and the data acquisition trigger was set on SYTO 13-associated fluorescence. This SYTO 13 threshold value was set sufficiently high such that the majority of blood cells (i.e., mature erythrocytes which lack RNA and therefore express low SYTO 13-associated fluorescence) was eliminated from consideration. This strategy provided greater rates of RET analysis, making it feasible to evaluate 1×10^6 RETs per specimen (typically ≤ 10 minutes).

2.7 Calculations

The incidence of *Pig-a* mutation is expressed as the number of CD24-negative RBCs per one million RBCs, and as the number of CD24-negative RETs per one million RETs. All frequency, average, and standard error calculations were performed with Excel Office X for Mac® (Microsoft, Seattle, WA). In order to evaluate the longitudinal ENU data, a non-parametric Kruskal-Wallis test was performed, and a p value ≤ 0.05 was used to indicate significance.

3. Results

3.1 Method Optimization

Initial experiments focused on the development of simple and reproducible methods for staining and analyzing peripheral blood cells for the GPI anchor-deficient phenotype. As reported elsewhere, we found that the GPI-anchored protein CD24 is highly and stably expressed on erythrocytes throughout their lifespan in circulation [9]. A second fluorescent reagent was chosen to differentially stain mature erythrocytes, RETs, and leukocytes. Whereas we had previously used thiazole orange for this purpose [1], more recent work indicated that SYTO 13 is superior in at least two respects. Firstly, it provided a more stable fluorescence signal that did not shift over time. Secondly, it provided better fluorescent resolution between RNA-negative RBCs, RETs, and blood leukocytes. The separation of these latter two populations is particularly important, since not all leukocytes express appreciable levels of CD24 [9], and these cells can therefore be mistaken for GPI anchor-deficient RBCs if they are not excluded from analysis. Early staining trials included a third fluorescent reagent to positively identify platelets. This anti-platelet antibody (biotinylated anti-CD61 plus

streptavidin-Cy5-PE) was added to prevent platelets from interfering with our RBC/RET-based scoring system.

The 3-color staining strategy outlined above appeared very promising, and resulted in very low mutant frequencies within the expected order of magnitude. Even so, we hoped to simplify the flow cytometric scoring procedure by eliminating the need for the anti-platelet reagent if possible. Indeed, we found that Lympholyte-based separation was sufficient to eliminate the vast majority of platelets and leukocytes from the RBC pellet. Samples processed in this manner were evaluated with and without the anti-platelet antibody, and virtually identical values were consistently obtained (data not shown). Thus, the new standard blood processing method consisted of the simpler 2-fluorochrome scheme described in Materials and Methods.

Figure 1 illustrates the gating strategy that was used to score GPI anchor deficient RBC populations. The middle panel illustrates the SYTO 13 fluorescence profile (Y axis) that is exhibited by mature versus young RBCs, as well as residual leukocytes. Gated events (RBCs) were then applied to a SYTO 13 vs anti-CD24-PE bivariate graph as illustrated in Figure 2. In addition to showing representative blood from vehicle control and mutagen-treated mice, this figure also demonstrates our use of an instrument calibration standard.

Despite the high throughput capacity of modern flow cytometers, the interrogation of approximately 10^6 RETs for mutation is time consuming. Given the RNA content of RETs and their higher SYTO 13 fluorescence intensity, it was possible to restrict analyses to RETs by using a sufficiently high fluorescence threshold to trigger data acquisition (Fig 3). In combination with an increased sample throughput rate, much quicker data acquisition rates are achieved. In this manner, through sequential analyses of the same stained specimen, it was possible to efficiently evaluate 10^6 RBCs and then 10^6 RETs.

Once the staining methodology had been optimized, the analytical performance of the system was evaluated in several manners, primarily through reconstruction experiments. Representative RBC data are graphed in Figure 4. In three independent experiments, the correlation coefficients (r^2) for observed and expected values were ≥ 0.9795 . Similar results for RET-based analyses were also obtained (data not shown).

3.2 DMBA

Two weeks following DMBA treatment, the frequencies of CD24-negative RBCs and RETs were determined. As shown in Fig 5, markedly elevated CD24-negative values were evident for each of the DMBA-exposed mice. Note that the responses in the RET cohort were consistently higher than those in the RBC population (mean frequencies for RBC and RET populations = 133×10^{-6} and 286×10^{-6} , respectively). This discrepancy may be due to the fact that the RBC pool would not have completely turned over by the 2-week post-treatment time-point. Additionally, this result would be expected if GPI anchor deficient RBCs exhibit a reduced lifespan in circulation. In order to investigate these possibilities further, the kinetics and persistence of mutant erythrocytes were studied further, as described below.

3.3 ENU

Blood specimens were collected at weekly intervals following ENU treatment. Figure 6 shows the results of this time-course study. Regarding the RET population, markedly elevated CD24-negative frequencies were observed for each of the 20 ENU-treated mice relative to controls. The magnitude of the effect was very large, as the average frequency was $14 \pm 21 \times 10^{-6}$ for vehicle-treated mice, and $500 \pm 287 \times 10^{-6}$ for the mutagen-exposed animals (weeks 1 – 5 pooled). Interestingly, significant changes over baseline values were observed quickly, that is, as soon as one week post-dosing. Although the average frequency of GPI anchor deficient

RETs was observed to drop at 3 weeks, this fluctuation is likely related to inter-animal variation and our use of different groups of animals for each time-point. In any event, mean mutant frequencies were not statistically different over weeks 1 – 5 ($p = 0.5319$).

Elevated frequencies of GPI anchor deficient RBCs were delayed relative to the RET cohort, as the first clear responses in the total RBC pool were not observed until the 2 week time-point. Thereafter, as with the RETs, the frequency remained elevated and relatively constant (no significant differences over weeks 2 – 5; $p = 0.4952$). Interestingly, the frequency never reached the level attained by the RET cohort, even after 5 weeks, a period of time that is sufficient for the entire pool of circulating RBCs to be replaced.

4. Discussion

Previous work with rat blood specimens suggested that platelets and leukocytes can interfere with RBC- and/or RET-based analyses, and for this reason a 3-fluorochrome staining strategy had been employed. Data presented herein suggest that a simpler staining method is possible, at least in the case of mouse blood. In this alternate scheme, leukocytes and platelets are first depleted with a Ficoll-type reagent. Then, to the extent that leukocytes continue to contaminate RBC specimens, they are further excluded based on a high nucleic acid dye-associated signal. Residual platelets are excluded based on their low light scatter profile. Data presented herein strongly suggest that by measuring the frequency of GPI anchor deficient RBCs and/or RETs in this manner, it is possible to detect chemical-induced mutation.

Given the conserved nature of the *Pig-a* locus, one would predict that it should be possible to apply flow cytometric approaches to *in vivo* mutation scoring across species of toxicological interest. Indeed, work with rats [1,4–5] and now mice strongly support this concept. Thus, in addition to other favorable characteristics of *Pig-a* based mutation assays (blood-based endpoint, persistent mutant phenotype, compatible with on-going toxicology studies, no cell culture requirement, objective scoring platform), its ability to bridge preclinical models has now been demonstrated.

The time course data presented herein are particularly interesting and relevant to discussions regarding the choice of dosing and harvesting schedule(s) that would be most appropriate for *Pig-a* mutation measurements. Specifically, these data show that the elevated mutant frequencies following genotoxicant exposure persist for at least five weeks, even for the RET cohort which turns over very quickly (days). This is in agreement with our previously reported rat data [1]. We interpret this to mean that the *Pig-a* mutant bone marrow erythrocyte progenitors or stem cells are not severely selected against *in vivo*, or the mutant frequency values would have diminished over time. This characteristic implies that *Pig-a* mutations are “neutral”. Keller *et al.* [9] and Rosti [10] reported on murine models of PNH, and provide additional evidence that *Pig-a* mutant erythroblasts are neither selected against, nor exhibit a marked growth advantage. With this characteristic in mind, one would predict that assay sensitivity will benefit from repeat dosing experiments, as mutant yields are likely to accumulate in an additive or at least nearly additive manner as they do for other neutral loci [12–13].

It should be noted that even if selection for or against *Pig-a* mutant stem cells and/or erythroid progenitors proves to be minimal, this is not necessarily the case for circulating RBCs that exhibit the GPI anchor deficient phenotype. Indeed, the higher mutant frequencies in RETs compared to RBCs reported here supports this premise, as does the prominent clinical feature of PNH, hemoglobinuria. While a strictly RBC-based mutation assay would be technically simpler and more rapidly performed relative to RET-based analyses, at this time it appears that further study of both populations is warranted.

In conclusion, the data presented herein support the further development and validation of *Pig-a* based mutation assays. As a blood-based endpoint that does not require transgenic or otherwise specialized animals, it would be relatively simple to integrate these measurements into on-going toxicology studies. Clearly though, further work is needed to establish the most appropriate dosing/harvesting schedule(s), evaluate additional genotoxicants and non-genotoxicants, and to test the portability of the method. Inter-laboratory studies are planned to address these gaps. Furthermore, it will be important to continue evaluating the degree to which GPI anchor deficiency is equivalent to *Pig-a* mutation. Regarding this last point, work is planned to expand mouse bone marrow erythroid progenitors that exhibit the GPI anchor deficient phenotype for *Pig-a* sequencing. Ultimately, assuming data from preclinical models continue to show promise, work with human specimens will be needed in order to understand the degree to which *Pig-a* mutation assays can provide genotoxicity data in human biomonitoring and/or clinical trial settings.

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Figure 1.

Three bivariate graphs illustrate the gating logic used for the mutant scoring application described herein. Only RBCs are plotted in the bivariate graph to the right. Other events are excluded based on their failure to exhibit light scatter characteristics of cells (left panel), or else their high SYTO 13 fluorescence intensity (middle panel, “Leukocytes”). Thus, while the Lympholyte reagent physically removed the bulk of platelets and leukocytes, this gating strategy further ensures that mutant RBC frequencies generated by the right plot are not affected by these spurious events.

Figure 2. Gated events (RBCs) are plotted on SYTO 13 versus anti-CD24-PE bivariate plots

Left: Instrument calibration standard; mutant-mimicking cells have been spiked into blood that was processed according to the standard protocol. This specimen provides enough events with a full range of fluorescence intensities to optimize PMT voltages and compensation settings. This calibration standard also represents a means for rationally and consistently setting the position of the vertical line that defines mutant versus non-mutant cells.

Middle: Vehicle control mouse blood; note the very low incidence of cells that appear in the upper left and lower left quadrants. These anti-CD24-negative events are presumably *Pig-a* mutant reticulocytes and RNA-negative RBCs, respectively.

Right: Blood from a mutagen (DMBA) treated mouse; note the elevated numbers of events that appear in the upper left and lower left quadrants, i.e., presumably *Pig-a* mutant reticulocytes and RNA-negative RBCs, respectively.

Figure 3.

Bivariate graphs illustrate analyses based on Forward Scatter thresholding (left panel), and SYTO 13 fluorescence thresholding (right panel). Forward Scatter thresholding is capable of efficiently evaluating 10^6 or more RBCs for the mutant phenotype, whereas SYTO 13 thresholding facilitates interrogation of 10^6 RETs.

Figure 4.

Expected number of CD24-negative RBCs per 10^6 total are plotted against observed frequencies measured by flow cytometry. The high correlation coefficient, coupled with the system's ability to differentiate control (unspiked) specimens from those with low numbers of mutant cells spiked in (< 100) suggest that analytical performance is sufficient for an *in vivo* mutagenesis assay.

Figure 5.

The number of presumed mutant cells are graphed for each of three vehicle (V) and DMBA (D75) treated mice. These two week post-dosing blood specimens exhibit higher mutant frequencies for the RET population (black bars) compared to the total RBC pool (white).

Figure 6.

Average mutant frequencies are graphed versus time post-ENU treatment. Whereas the mutant RET frequency is elevated as soon as week 1, the response in the total RBC cohort is delayed. Note that there were actually no “time zero” bloods, rather this data point was generated by pooling vehicle control specimens that were obtained throughout the experimental time-frame (one per time point, for a total of 5 observations).