

## COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

### The Development and Validation of a Mutation Assay using the PIG-A gene

#### Background and general principles of the assay.

1) To date there has been limited success at the development and application of assays to study somatic mutations in animals and in man. Most robustly assessed is the use of the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene, in which thymidine kinase deficient mutations are selected for in appropriate medium (Albertini et al 1990). However this assay has not been widely applied for the analysis of *in vivo* mutagenesis as it requires lengthy and labour intensive *in vitro* culture methods for mutation detection and has a limited sensitivity. The use of transgenic animals such as in the Big Blue and MutaMouse assays has been more widely utilised and accepted as an *in vivo* screen for mutation (COM/05/S6 and COC/05/S3) but these also have the limitations of expense, labour intensiveness and also the disadvantage that they are not transferable to measuring mutation in humans.

2) More recently the PIG-A gene, ubiquitous in mammals, has been identified as a potentially useful gene for the study of somatic mutation rates and mutations induced by chemicals *in vivo*. The PIG-A gene codes for one subunit of a glycosylphosphatidyl inositol (GPI) anchor protein and it was discovered that mutations in the PIG-A gene in humans are responsible for the disease paroxysmal nocturnal hemoglobinuria (PNH) (Bessler et al 1994). It was recognised as having the potential to be a good sentinel gene for the study of somatic mutations for a number of reasons: PIG-A is located on the X chromosome which means that a single mutation results in an altered phenotype, all mutant cells are viable and the mutation does not result in enhanced or reduced growth (i.e growth neutral) (Peruzzi et al 2010). Furthermore, as the GPI gene product function is to tether other proteins to the cell surface, it was quickly established that loss of function arising from PIG-A mutation could readily be assessed using straightforward immunochemistry and flow cytometric methods looking for the presence or absence of these GPI linked proteins (Alfinito et al 1996). The GPI linked proteins which have been identified as being potential candidates for detection of mutation in PIG-A include CD59, CD55 and CD48; these proteins are easily identified using well established immunotechniques. Taken together these factors indicated the utility of PIG-A as a gene for the detection of the somatic mutation rate and the incidence of induced mutations *in vivo*. It was predicted that it could be applied to a variety of different mammalian species including man.

3) Although PIG-A is ubiquitously expressed, selection of a cell type to develop a viable assay was not straightforward. GPI-linked proteins CD59 and CD55 are key regulators of complement activation. Therefore in PNH patients GPI reticulocytes (RET) are very sensitive to complement and are selectively removed from the circulation by lysis. It has also been established that mouse red blood cells (RBC)

have a reduced lifespan because of this increased sensitivity. In granulocytes, although there is no evidence that GPI<sup>-</sup> cells have a shorter life-span than GPI<sup>+</sup> cells, there are concerns regarding the potential for a mutation to occur in the stem cell. This is likely to impact on the time course of appearance of mutations as well as the absolute number of mutant cells if the damage occurred at different stages of cell maturation. However, it seems that experience and success with the use of red blood cells for the screening and analysis of PIG-A mutations in PNH patients has directed the development of the assay for screening purposes in this direction. Furthermore, the ease with which red blood cells can be sampled adds to its attractiveness for use in routine screening.

4) Analysis of PIG-A mutation has been used to effectively determine the rate of mutation ( $\mu = f/d$ , where  $f$ , the frequency of cells with mutation, and  $d$  the number of cell divisions) (Araten et al 2005). B-lymphoblastoid cells were stained with an antibody which recognises the GPI linked protein CD59. Cells were sorted using flow cytometry, enabling the elimination of pre-existing mutants cells. Following 2 weeks in culture cells were assessed by flow cytometry to determine  $f$ . This was done by staining with a mixture of antibodies, CD59, CD55 and CD48, all GPI linked proteins. Mean  $\mu$  values of  $3.4 \times 10^{-7}$  /cell division in cell lines from normal donor and  $110 \times 10^{-7}$  in ataxia telangiectosiam donors. It was concluded that the PIG A<sup>-</sup> phenotype can be clearly identified by flow cytometry and it was also suggested that there is a capacity to measure the relationship between  $\mu$  and cancer.

#### **Proof of concept studies**

5) Developmental and validation of the potential use of the PIG-A assay has thus far been confined essentially to two laboratories (Dertinger et al, Litron Laboratories, Rochester NY, USA and Miura et al, (TEIJIN Pharma, Tokyo, Japan)

6) Miura and colleagues (Miura et al 2008a) conducted a study which can be considered to be a key preliminary investigation to establish the basic methodological parameters for the assay. The aim of this specific study was to design appropriate conditions for the identification of GPI-deficient cells in peripheral blood and splenic T-cells of mutagen treated rats. Ethyl nitrosourea (ENU) was administered at 40mg/kg intraperitoneally (ip) every other day for 3 days (total 120 mg/kg) to male F344 rats. Peripheral blood was sampled 2 and 4 weeks after dosing. Spleen cells were isolated and treated to be suitable for flow cytometry. CD59 and CD48 GPI-anchored markers were used as reporters of PIG-A in RBC and T- cells respectively. CD-45 antibody is also used - cells negative for CD-45 were gated and excluded from the analysis (assumed to be platelets). In addition FLAER, a fluorescent tag specifically binding GPI anchors was used to detect GPI mutants.

7) Flow cytometry plots were gated to exclude white blood cells and platelets. Control mean frequency of CD59 negative cells ranged from 1 to  $27 \times 10^{-6}$  cells. Prior to ENU exposure these rats had MF of 5 to  $71 \times 10^{-6}$  and then following treatment this increased to  $219 \pm 29 \times 10^{-6}$  at 2 weeks to  $375 \pm 32 \times 10^{-6}$  at 4 weeks. In the spleens, CD48 negative cells were measured and showed that MF increased from  $13 \pm 2 \times 10^{-6}$  in controls to  $402 \pm 171 \times 10^{-6}$  in ENU treated rats. The authors established that it was of importance to gate accurately; excluding platelets and WBC's was considered essential. It was shown that it was easy to differentiate

between the cell types as they caused differing forward and side scatter plots. It was concluded that it was most useful to examine RBC's and that this method requires only a small amount of blood allowing for regular non-invasive sampling. However it is pointed out that as the RBC's lack DNA it is not possible to positively confirm the GPI-deficient phenotype.

8) In a second study the same authors examined spleen cells for PIG-A mutations (Miura 2008b). Four weeks after treatment with ENU (dosing schedule as Muira et al 2008a), splenocytes were isolated from rats and cultured for 10 days. Cells which became resistant to proaerolysin (ProAER) and formed colonies were considered to be GPI-deficient. PIG-A was also analysed using CD48 antibodies and mutated genes from the ProAER deficient colonies were sequenced. Dosing with ENU increased the number of ProAER cells by more than 100-fold. It was concluded that this offered a rapid and straightforward assay to evaluate mutations *in vivo*.

9) Another preliminary study examining the practicality of using the analysis of PIG-A mutations as a genotoxicity screen was undertaken by Bryce et al (2008) from Litron Laboratories. Groups of female Sprague Dawley rats were administered ENU (i.p. at 100 mg/kg/day) or DMBA (p.o. at 40 mg/kg/day) a total of three times, every other day (ie on days 1, 3 and 5) and blood was collected 1, 2, 4 and 5 weeks after the last dose. Appropriate groups of negative controls were included. After counting,  $10^8$  cells were incubated with a saturating level of CD59-PE antibody. Biotinylated anti-CD-61 was used to identify platelets. Thiozole orange was used to differentially stain RBC and RET's and then flow cytometry was used to identify CD-59<sup>-</sup> in both cell populations.

10) The incidence of PIG-A mutations was expressed as the number of CD59<sup>-</sup> /  $10^6$  RBC or RET. Very large increases in the frequency of mutant were measured following both ENU and DMBA administration. Increases were noted 1 week after treatment was initiated, and maximal responses were apparent approximately 4 weeks after treatment. The increases were apparent in both RBC and RET, the mutation frequency generally being greater in the RET. The short time frame needed to express the mutant phenotype in RET's was stated as a reason that RET's had an advantage over RBC's. It was concluded that the PIG-A assay shows promise for the identification of *in vivo* mutations, and that it has the clear advantages of being easily incorporated into routine toxicity studies as blood samples can be readily collected.

### **Method development studies**

11) Miura et al (2009) extended their initial work by examining the hypothesis that the mutation frequency (MF) will be maintained over an extended period of time.

Male F344 rats were dosed as follows: with either

a) Controls: 4 weekly doses of vehicle

b) ENU single dose: a single dose at 8.9, 35.6 or 142.4 mg/kg

c) ENU multiple doses: 4 weekly doses of 8.9 or 35.6 mg/kg (totalling 35.6 and 142.4 mg/kg)

12) Peripheral blood was collected 1, 2, 3, 4, 6, 9, 13, 16, 20 and 26 weeks after the first dose. Flow cytometry was used to detect CD59 negative cells after gating for CD45 negative cells (staining as described in para 6). It was shown that mutations continued to increase for up to 9 weeks after dose administration in all groups and in general were maintained at these maximum levels for the rest of the period examined. Dose related increases were seen - the control range at 6 weeks was  $3.9-21.4 \times 10^{-6}$ , with maximal responses of 57.3, 171.7 and  $624.6 \times 10^{-6}$  for 8.9, 35.6 and 142.4 mg/kg respectively. It was shown that splitting the dose into four smaller doses resulted in very similar final MF's. At the high dose, there was large inter-animal variation after the maximum response had been observed - speculated to be a result of differing rates of decline/maintenance of mutants in some animals or disproportionate expansion of mutant cells in some animals potentiated by the toxic stress induced by ENU in the bone marrow.

13) Phonethswath et al (2008b) aimed to demonstrate the utility of the PIG-A gene in a different species, namely mice. Female CD-1 mice (n=3) were treated with DMBA at 75 mg/kg or ENU at 40 mg/kg, three times at 2 day intervals. Blood was sampled weekly for 5 weeks and mutations in PIG-A detected using anti-CD24-PE in RBCs and RET's. Further refinement and optimization of the method was undertaken as in this study the use of CD24 was demonstrated to improve fluorescence resolution. Following DMBA treatment at 2 weeks, mutation frequency was significantly increased (only time point shown) and after ENU treatment, MF was significantly elevated from 2-5 weeks. The greatest increase was from  $14 \pm 21 \times 10^6$  to  $500 \pm 287 \times 10^6$ . The MF was again higher in the RET's compared to the RBC's ( $286 \times 10^6$  compared to  $133 \times 10^6$  for DMBA), and increased more rapidly. It was discussed that the capacity of the flow cytometry to analyse  $10^6$  RET's was time consuming. However it is noted that further investigation of the assay using both cell types is warranted.

14) A recent study has extended the number of compounds studied and also investigated the use of two different strains of rat (Phonethpswath et al 2010). Groups of male Wistar rats (n=5) were administered orally ; ENU (at 20 and 40 mg/kg), DMBA (25 and 50 mg/kg), 4-nitroquinoline-1-oxide (NQO; 12.5 and 25 mg/kg), benzo[a]pyrene (BaP; 125 and 250 mg/kg) or N-methyl-n-nitrosourea MNU (15 and 30 mg/kg). Sprague Dawley rats were also administered ENU. These doses were administered daily for 3 days. Blood was taken on day -1, 4, 15, 30, 45 and 90 days post the last dose. CD59-PE and SYTO 13 anti-bodies were used to score number of CD59<sup>-</sup> RBC and RET using flow cytometry. For ENU an extended time course, examining mutation at 6 months, was also included.

15) All chemicals induced increases in mutated cells (CD59<sup>-</sup>) in both RET and RBC although it is noteworthy that MNU did not induce a strong response. However the time response patterns differ quite significantly for different chemicals. Maximal responses varied; at 6 weeks for ENU compared to 2 weeks for NQO, with responses more rapid in RET's compared to RBC's as demonstrated in other studies. Similarly robust response were noted in the two strains of rat for ENU. Over the 6 month time period, MF's were seen to decrease in RET's but remained elevated in RBCs. Overall the authors conclude that the PIG-A assay will provide an efficient way of measuring *in vivo* mutations and the kinetics of the mutations in RBC's and RETs means that it can easily be incorporated into routine toxicology studies.

16) The potential to use peripheral blood taken from non-human primates during routine toxicology studies has also been examined (Dobrovolsky et al 2009). In the study both PIG-A and HPRT were evaluated. Animals on a study assessing an electrolyte replenisher (Prang) were examined for mutations and also one male *Macaca mulatta* monkey had received a single i.p dose of N-ethyl-N-nitrosourea (40 mg/kg) at 2 years of age and again at 3.5 years of age. Blood was taken approximately 3 months after the second dose. Blood was labelled with anti-human CD59 and PE-Cy5-labeled anti-human CD45 and analysed by flow cytometry with appropriate gating. Control values for PIG-A mutations was  $7.8 \pm 4.2 \times 10^6$  (range 2.2-13.3). In the bloods from the treated animal the MF value was  $46.5 \times 10^6$ . Furthermore, several methodological aspects of labelling were explored in the control samples. This study gives an early indication of the how the PIG-A assay has the potential to be transferable to a number of other species.

17) Dobrovolsky et al (2010) also used the assay to investigate methylphenidate (MPH), a drug which is reported to increase cytogenetic endpoints in treated children. MPH was given at 3 mg/kg, split into doses of approx 1 mg/kg 3 times daily to adolescent rats (post natal day PND21, males and females, based on a therapeutic dosing regimen); ENU at 65 mg/kg was included as a positive control, was given as a single dose on PND28. Blood was collected on PND29 and 50. Two labelling techniques were used to detect PIG-A mutations using CD59 antibody (TH9 clone); firstly with mouse anti-rat CD45 antibody (clone OX-1) and then with HIS49 antibody. Both labelling methods were subjected flow cytometric analysis.

18) ENU induced clear increases in CD59<sup>-</sup> mutants in both male and females and using both labelling methods. MPH did not cause any elevation in MF.

Scores as CD59 <sup>-</sup> mutant RBC's x 10 <sup>-6</sup>				
Treatment	Males		Females	
	CD45	HIS49	CD45	HIS49
Control	14.9 ± 7.5	6.1 ± 3.8	11.1 ± 6.8	4.6 ± 3.5
MPH	15.6 ± 10.7	6.4 ± 5.9	12.7 ± 9.9	3.3 ± 3.3
ENU	286.7 ± 98.8	310.0 ± 114.8	210.4 ± 60.7	195.8 ± 53.9

19) Dertinger and colleagues (2010) have also assessed the ease with which assessment of PIG-A mutations can be incorporated into a standard 28 days toxicity study. All chemicals were given daily for 28 days as follows N-ethyl N-nitrosourea (2.5, 5 and 10 mg/kg/day), DMBA (2.5, 5 and 10 mg/kg/day), NQO (1.25, 2.5 and 5 mg/kg/day), BaP (37.5, 75 and 150 mg/kg/day) and N-methyl nitrosourea (2.5, 5 and 10 mg/kg/day). These dose levels were all show not be maximum tolerated doses for this administration duration. Flow cytometric scoring of CD59 negative RBC's was undertaken on days -1, 15, 29 and 56. All chemicals induced dose -related increases in MF in both RBC's and RET's and most MF's continued to increase after dosing stopped (ie at the 56 days timepoint). However again there is variability; for example DMBA caused little response in RBC's but a huge increase in RET's.

20) The authors commented that despite previous demonstration of mutation accumulation, that the cumulative smaller doses of e.g MNU induced less mutations

than a smaller number of larger doses. This is possibly explained by the instigation of effective DNA repair for low doses which is overwhelmed by larger doses. This study also examined the effects of aerolysin, a high affinity GPI anchor ligand that causes cell lysis

### **Discussion;**

21) A number of studies have been conducted and reported in which the PIG-A gene has been utilised to assess gene mutations *in vivo*. It has been shown to be feasible in a number of different animal species and in different blood cells and in splenocytes. The relative ease with which the GPI linked proteins can be labelled using conventional techniques and sorted and scored using flow cytometry (FACS) has greatly facilitated the development of an assay using PIG-A mutation as an endpoint. From these preliminary studies it is clear that much effort has been directed at investigating the use of different anti-bodies and FACS conditions. However, as yet it seems that no definitive protocol has emerged. Nevertheless, these studies show the prospective utility of the assay. Furthermore, potentially it can be performed as an adjunct to rodent toxicity studies since blood samples can be taken and it is likely that it can be directly transferable to man, so that the measurement of mutation in epidemiology studies is also a promising possibility.

### **Questions for Members:**

- What are Members opinions on the general development and utility of assay?
- Do the Members have any comments on the specific methodologies (e.g. choice of different anti-bodies, use of RET vs RBC?)
- What do Members consider to be further validation requirements?
- How could this assay be incorporated into the COM testing strategy?

**DH Toxicology Unit/Secretariat  
May 2010**

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